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(54) Title: VACCINE COMPOSITIONS AND METHODS OF MODULATING IMMUNE RESPONSES

(57) Abstract

Methods of modulating an immune response are disclosed wherein a fusion protein comprising an antigen and an APC binding domain of an opsonin or a nucleic acid encoding a fusion protein comprising an antigen and an APC binding domain of an opsonin is administered as an immunogen.

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VACCINE COMPOSITIONS AND METHODS OF MODULATING IMMUNE RESPONSES

Field of the Invention

This invention relates to vaccines useful, for example, for modulating immune responses in subjects to a variety of antigens.

Background of the Invention

The innate immune system comprises those mechanisms that have evolved over millennia to provide first line defense against foreign antigens and an antigen recognition repertoire which does not diversify during the ontogeny of the individual. This is in contrast with the acquired immune system which provides later phase defense mechanisms and depends on a repertoire of antigen specific molecules, e.g., immunoglobulins and T cell receptors that diversify over the ontogeny of the individual. Innate immune mechanisms can contribute to initiation of an antigen specific response by the acquired immune system, for example by facilitating uptake of antigen by antigen presenting cells (APCs), which can thereafter stimulate cognate T cells.

Opsonins of the innate immune system ("innate opsonins") are known in the art as secreted polypeptide molecules of the innate immune system and can remain bound to an antigen and to the surface of an APC at the same time. They can thus act as "bridges", and are thought, by virtue of this property, to promote internalization of antigens by APCs. The mode in which opsonins bind to antigens varies among opsonins, and can be covalent or noncovalent. In general, the antigen binding moieties of innate opsonins differ from the antigen binding moieties of immunoglobulins in that the former are relatively invariant among members of the same species, and do not undergo diversification during the ontogeny of an individual.

There have been a number of attempts to increase uptake of antigens by APCs by coupling an antigen via a nonpeptide linkage to another molecule that can bind to the surface of an APC. Targeting moieties have included, for example, C3b (JacquierSarlin et al., *Immunol* 84:16470; Arvieux et al., *Immunol* 65:22935), alpha2 macroglobulin (Chu et al, *J Immunol* 152:153845; Chu and Pizzo, *J. Immunol.*, 150:4858), and molecules comprising idiotypes specific for immunoglobulin Fc receptors (Squire et al., *J Immunol.* 152:438896; Gosselin et al., *J Immunol.* 149:347781; Snider and Segal, *J Immunol.* 143:5965) or class II MHC molecules (Estrada et al., *Vaccine* 13:9017; Berg et al., *Eur J Immunol.* 24:12628; Carayanniotis and Barber, *Nature* 327:5961).

Another approach to improving uptake of antigen by APCs has been to construct chimeric

polypeptides comprising an antigen and an idiotypic portion of an antibody, in which the latter is specific for class II MHC molecules (Baier et al., *J Virol* 69:235765) or an immunoglobulin Fc receptor (Liu et al., *JCI* 98:20017).

Dempsey et al. (*Science* 271:348 50) constructed fusion proteins between C3d and an antigen, the fusion proteins being capable of binding to CR2-bearing cells such as B cells, reasoning that the B cell costimulation provided by C3d would increase the humoral immune response to the antigen. These fusion proteins do not bind to antigen presenting cells. Marked increases in antibody response were in fact observed, which were abrogated by *in vivo* antibody blockade of CR2.

Summary of the Invention

The invention provides compositions and methods for modulating immune responses in subjects. The invention is based, at least in part, on the discovery that an in-frame translation fusion of an antigen with a cell binding domain of a ligand for a cell-surface polypeptide, e.g., an APC binding domain of an opsonin, forms a molecule, that is, a fusion polypeptide, which when administered to a subject modulates an immune response to the antigen.

The invention thus encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising an antigen and an APC binding domain of an opsonin in an amount and for a time effective to modulate said immune response.

The invention also encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising an antigen and an opsonin in an amount and for a time effective to modulate said immune response.

The invention also encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising an antigen and a first portion of an opsonin which when associated with a second portion of said opsonin forms an APC binding domain or a multichain polypeptide complex comprising (a) a fusion polypeptide comprising an antigen and a first portion of an opsonin which when associated with a second portion of an opsonin forms an APC binding domain, covalently associated with (b) said second portion in an amount and for a time effective to modulate said immune response.

In all of the above-described inventive methods, the opsonin may be drawn from the group: fibronectin, alpha2macroglobulin (a2m), c-reactive protein (CRP), complement component C1q,

complement component C3, complement fragment C3b, complement fragment C4b, mannose binding protein (MBP), conglutinin, and surfactant proteins A and D. The antigen may be drawn from the group: an antigen of a bacterium, an antigen of a virus, an antigen of a fungus, an antigen of a parasite. The antigen also may be drawn from the group: an antigen involved in (i.e., which promotes) autoimmune disease, an antigen involved in allergy, an antigen involved in graft rejection. Alternatively, the antigen may be a tumor antigen.

The invention also encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising a secretory signal sequence, an antigen, a first cell binding domain of a ligand for a cell surface polypeptide, and a second cell binding domain of a ligand for a cell surface polypeptide in an amount and for a time effective to modulate said immune response.

In the above methods, the first and second cell binding domains are identical, or the first and second cell binding domains may be nonidentical.

The first and second cell binding domains bind to the same cell surface polypeptide or the first and second cell binding domains bind to different cell surface polypeptides.

In this embodiment, it is preferred that neither the first nor the second cell binding domain comprises the antigen.

Other preferred embodiments include: at least one of the cell binding domains is endogenous (i.e., naturally expressed in) in the animal; at least one of the cell binding domains is endogenous to (or naturally expressed in) a eukaryotic cell; at least one of the cell binding domains may be an APC binding domain of an opsonin.; the antigen may comprises greater than 7 and no more than 25 amino acids.

The invention also encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a fusion polypeptide comprising an antigen and an APC binding domain of an opsonin in an amount and for a time effective to modulate said immune response.

The invention also encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a fusion polypeptide comprising an antigen and an opsonin in an amount and for a time effective to modulate the immune response.

The invention also encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a fusion polypeptide comprising an antigen, a first cell binding domain of a ligand for a cell surface polypeptide which is an APC binding domain of an opsonin, and a second cell binding domain of a ligand for a cell surface polypeptide

in an amount and for a time effective to modulate the immune response.

The invention also encompasses a method of modulating in an animal an immune response to an antigen, comprising administering to the animal a fusion polypeptide comprising an antigen, an opsonin, and a cell binding domain of a ligand for a cell surface polypeptide in an amount and for a time effective to modulate the immune response.

In these methods, the opsonin may be drawn from the group: fibronectin, alpha2macroglobulin (a2m), c-reactive protein (CRP), complement component C1q, complement component C3, complement fragment C3b, complement fragment C4b, mannose binding protein, conglutinin, and surfactant proteins A and D.

The antigen also may be drawn from the group: an antigen of a bacterium, an antigen of a virus, an antigen of a fungus, an antigen of a parasite.

The antigen also may be drawn from the group: an antigen involved in autoimmune disease, an antigen involved in allergy, an antigen involved in graft rejection.

The antigen also may be a tumor antigen.

The invention also encompasses an isolated nucleic acid comprising a nucleotide sequence encoding a fusion polypeptide comprising an antigen and an APC binding domain of an opsonin, the opsonin being drawn from the group: c-reactive protein (crp), complement component C1q, complement component C3, complement fragment C3b, complement fragment C4b, a collectin, mannose binding protein, conglutinin, and surfactant proteins A and D.

In preferred embodiments, the APC binding domain of an opsonin comprises an opsonin; the APC binding domain is an APC binding domain of mannose binding protein that does not contain a lectin domain or a complement-fixing domain.

The invention also encompasses an isolated nucleic acid comprising a nucleotide sequence encoding a fusion polypeptide comprising an antigen and an APC binding domain of alpha-2macroglobulin and the antigen is neither a portion of the adenovirus fiber protein nor carbonic anhydrase nor a heptapeptide which comprises a cleavage site for the TEV protease

In preferred embodiments, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a fusion polypeptide comprising an antigen wherein the antigen is drawn from the group: an antigen of a bacterium, an antigen of a fungus, an antigen of a parasite; wherein the antigen is an antigen of a virus; wherein the antigen is an antigen of a virus that infects animal; wherein the antigen is drawn from the group: an antigen involved in autoimmune disease, an antigen involved in allergy, an antigen involved in graft rejection; wherein the antigen is a tumor antigen; wherein the antigen comprises greater than 7 and no more than 25 amino acids.

Preferably, the isolated nucleic acid further comprising a sequence encoding a first portion of an opsonin which when associated with a second portion of the opsonin forms an APC binding domain.; wherein the fusion polypeptide consists essentially of an antigen and an APC binding domain of an opsonin; wherein the opsonin is mannose binding protein and the antigen is neither CD4 or a cytotoxin.

The invention also encompasses a polypeptide encoded by a nucleic acid as described above.

The invention also encompasses a multichain polypeptide complex comprising (a) a fusion polypeptide comprising an antigen and a first portion of an opsonin which when associated with a second portion of an opsonin forms an APC binding domain, covalently associated with (b) the second portion.

Preferably, in the multichain polypeptide complex of the opsonin is a collectin.

The invention also encompasses a composition comprising the above-described fusion polypeptide admixed with antigen presenting cells.

The invention also encompasses a composition comprising such a multichain polypeptide complex admixed with antigen presenting cells.

The invention also encompasses a vector containing a nucleic acid as described above, and a host cell transfected with the vector.

The invention also pertains to recombinant nucleic acid molecules which include a nucleotide sequence encoding an antigen and a nucleotide sequence encoding cell binding domain, e.g., an APC binding domain, and thus include a nucleotide sequence encoding a fusion polypeptide comprising the antigen and the cell binding domain.

As used herein, "modulation" means that a desired/selected response is more efficient, more rapid, greater in magnitude, and/or more easily induced than if the antigen had been used alone. The desired immune response can be stimulation/activation of a selected immune response, e.g., selective enhancement of an immune response to an antigen, or it can be inhibition of a selected immune response e.g., selective suppression, elimination, or attenuation of an immune response to an antigen, or a combination thereof.

According to the invention, a "cell binding domain" of a ligand for a cell surface polypeptide refers to the whole of a ligand for a cell surface polypeptide or that portion or domain of a ligand for a cell surface polypeptide that binds to a cell surface polypeptide.

As used herein, an "APC binding domain" refers to the whole of an innate opsonin or that portion or domain of an innate opsonin that binds to antigen presenting cells (APC binding domain). Thus, an APC binding domain is a type of cell binding domain.

In a fusion polypeptide according to the invention, different peptides or polypeptides are linked in-frame to each other to form a contiguous chimeric polypeptide. Thus, a first portion of the fusion polypeptide comprises an antigen and a second portion of the fusion polypeptide, either the amino- or carboxy-terminal to the first portion, comprises a functional ligand for a cell surface polypeptide, e.g., a functional opsonin moiety. It is critical in the fusion polypeptide that the antigen retain its antigenicity and the cell binding domain retains its ability to facilitate or permit binding of the fusion polypeptide to the cell; that is, the two portions of the fusion polypeptide must be able to assume their natural structure to the extent that they retain the antigenicity and binding functions necessary to modulate the immune response according to the invention. The amino and carboxy-terminal orientation of the antigen and the cell binding domain, e.g., the APC binding domain, will most likely be determined by the location of the cell binding domain in the ligand, e.g., the location of the APC binding domain in the opsonin molecule; that is, if the APC binding domain is located near the amino terminus of the opsonin, then the amino-terminal portion of the opsonin may correspond to the amino terminus of the fusion polypeptide; similarly, if the APC binding domain is located near the carboxy terminus of the opsonin, then the amino-terminal portion of the opsonin may correspond to the carboxy terminus of the fusion polypeptide.

Examples of categories of antigens which can be encoded by the nucleic acid molecules include, for example, viral antigens, bacterial antigens, fungal antigens, protozoal and other parasitic antigens, tumor antigens, antigens involved in autoimmune disease, allergy and graft rejection, and other miscellaneous antigens. As used herein, "involved in" refers to "promotes".

Cell binding domains of ligands for cell surface polypeptides can include, for example, cell binding domains of adhesion molecules, e.g., LFA-3, ICAM-1, or lectins, e.g., selectins such as L-selectin, E-selectin, AND P-selectin, cytokines, anaphylatoxins such as C3a AND C5a, costimulatory molecules such as B7-1, B7-2, and CD40, counterreceptors for costimulatory molecules such as CD28, CTLA-4, or CD40 ligand, and ligands for integrins. A cell surface polypeptide is any polypeptide that is naturally linked to the plasma membrane of a cell and, when so linked, at least part of which is located in the extracellular space. A cell surface polypeptide may be linked to the plasma membrane by, e.g., a transmembrane segment or a glycosylphosphatidylinositol moiety. A ligand for a cell surface polypeptide may bind to the polypeptide without binding directly to the amino acid sequence of the polypeptide, e.g., by binding to carbohydrates that are covalently attached to the cell surface polypeptide.

APC binding domains of innate opsonins encoded by the nucleic acid molecules can include, for example, an APC binding domain of fibronectin, a2macroglobulin (a2m), C-reactive protein

(CRP), complement component C1q, complement fragment C3b, complement component C4b, mannose binding protein (MBP), conglutinin, and surfactant proteins A and D.

The nucleic acid molecules of the invention can be used, for example, to modulate an immune response in a mammal to an antigen encoded by the nucleic acid molecule by direct administration of the nucleic acid.

Methods of the invention include the step of administering to an animal a nucleic acid molecule which encodes a fusion polypeptide comprising an antigen and a cell binding domain of a ligand for a cell surface polypeptide, e.g., an APC binding domain of an innate opsonin, or which encodes a polypeptide complex as defined herein, in an amount and over a period of time effective to modulate an immune response to the antigen in the mammal.

In one embodiment, the ligand is a lectin. A "lectin" is a polypeptide which can bind noncovalently to a carbohydrate. Typically, lectins bind selectively to certain carbohydrates, i.e., bind to those carbohydrates with higher affinity than to most other carbohydrates. In a preferred embodiment, the lectin is a selectin, e.g., L- selectin, E- selectin, OR P- selectin. In another preferred embodiment, the lectin is a collectin, e.g.,mannose binding protein.

In another embodiment, the ligand binds to a cytokine receptor. In yet another embodiment, the ligand is a counterreceptor for a costimulatory molecule, e.g., CD28, CTLA-4, or CD40 ligand. In a further embodiment, the ligand is a ligand for an integrin.

In the most preferred embodiment, the ligand is a ligand for an opsonin receptor and the cell binding domain is an APC binding domain.

It is most preferred that the nucleic acid encoding the fusion polypeptide further encode a secretory signal sequence which is linked in-frame to the fusion polypeptide.

In a preferred embodiment, the nucleic acid encodes two or more cell binding domains, neither of is identical to the antigen. The cell binding domains may bind to the same cell surface polypeptide or to different cell surface polypeptides. The cell binding domains may be identical to each other, e.g., multiple copies of the same opsonin, or they may be nonidentical.

In one embodiment, the nucleic acid encodes a fusion polypeptide that comprises an APC binding domain of an opsonin and a second cell binding domain of a ligand for a cell surface polypeptide. In a preferred embodiment, the second cell binding domain is also an APC binding domain of an opsonin. In another preferred embodiment, the second cell binding domain binds to a receptor for a cytokine, e.g., GM-CSF, IL-1, IL-4, IL-6, IL-10, IL-12, TNF-alpha, gamma interferon, or a chemokine. In yet another preferred embodiment, the second cell binding domain is a lectin. In still another preferred embodiment, the second cell binding domain binds to a receptor

for an anaphylatoxin, e.g., C3a or C5a. In a further preferred embodiment, the second cell binding domain binds to CD40.

It is preferred that a cell binding domain of a fusion polypeptide of the invention bind to a cell surface polypeptide that is selectively expressed on a leukocyte, or on an APC, or on a cell of monocytic lineage, or on a macrophage, or on a dendritic cell that is not a follicular dendritic cell, or on a B lymphocyte, or on a T lymphocyte, or on a follicular dendritic cell. A cell surface polypeptide is "selectively expressed" on a given cell type if it is naturally expressed on that cell type but is not naturally expressed on at least one of the following cell types: a cell of monocytic lineage, a macrophage, a dendritic cell that is not a follicular dendritic cell, a B lymphocyte, a T lymphocyte, a follicular dendritic cell, a neuron, a fibroblast, a rhabdomyocyte, a leiomyocyte, or a cardiomyocyte. Expression is easily discerned by techniques well-known to those skilled in the art, e.g., by staining with fluorescently tagged monoclonal antibody to the molecule in question and performing conventional flow cytometry analysis.

In the nucleic acids of the invention it is preferred that the region encoding the fusion polypeptide lack intronic sequences. It is particularly preferred that the region encoding the cell binding domain lack intronic sequences. According to the invention a DNA sequence which lacks intronic sequences is a "cDNA".

As used herein, an "animal" refers to a nonrodent animal, preferably, a nonrodent mammal, more preferably, a primate, and most preferably, a human.

The invention also pertains to vectors which include the nucleic acid molecules of the invention, host cells which are transfected with such vectors, and transgenic animals which include the nucleic acid molecules of the invention.

In another embodiment of the invention, where a first and second portion of an opsonin, when covalently associated via a non-peptide bond, form an APC binding domain, the first or second portion of the opsonin may be fused in-frame to the antigen to form a fusion polypeptide. The remaining second or first portion, respectively, may then be covalently associated with the fusion polypeptide via a natural mechanism in the host cell and form a complex.

Therefore, in another aspect, the invention pertains to a multichain polypeptide complex comprising a first portion of an innate opsonin which in the presence of a second portion of an innate opsonin forms an APC binding domain, and a fusion polypeptide that comprises an antigen and the second portion of the opsonin. The first portion of the innate opsonin is associated with the fusion polypeptide containing the second portion of the opsonin via a covalent linkage. Therefore, the polypeptide complex comprises at least two polypeptide chains which, when covalently

associated, form an APC binding domain. Furthermore, at least one of the chains may comprise an antigen.

Antigens and opsonins which can provide components of the fusion polypeptides are described herein.

In yet another aspect, the invention pertains to vaccine compositions. The vaccine compositions include nucleic acid molecules including nucleotide sequences encoding a fusion polypeptide or a polypeptide complex as described herein and a pharmaceutically acceptable carrier.

The vaccine compositions of the invention can be used for, for example, modulating in an animal an immune response to the antigen.

Methods of administering a vaccine according to the invention include the step of administering naked nucleic acid, which may be RNA or DNA, to the animal. As used herein, "naked" refers to nucleic acid which is substantially free from substances which facilitate entry of the nucleic acid into a host cell, for example, liposomes, ligands specific for cell surface receptors, endosomal disruption agents, etc.

Detailed Description of the Invention

Methods according to the invention pertain to administration to an animal of a composition which comprises a fusion polypeptide which includes an antigen fused to a cell binding domain, e.g., an APC binding domain for a time and in an amount sufficient to modulate an immune response to an antigen. The fusion polypeptide will bind to a cell surface polypeptide with a binding constant having an affinity that is at least in the nanomolar range, and the antigen itself (i.e., free of the fusion polypeptide) will not bind to the same APC or will bind at a significantly lower affinity (at least 10-fold lower).

A composition of the invention also comprises a multichain polypeptide complex which includes (a) a first portion of an opsonin which when covalently associated with a second portion of the same opsonin forms an APC binding domain and (b) a fusion polypeptide that includes an antigen and the second portion of the opsonin. That is, the fusion polypeptide will include an antigen and at least a portion of an opsonin that, when associated with an APC binding moiety of an innate opsonin, permits binding of the composition to an APC. As used herein, "associated with" refers to covalent bonding which is not peptide bonding, the covalent bonding being, for example, disulfide or hydroxyl bonding. The complex also will bind to an APC with a binding constant having an affinity that is at least in the nanomolar range, and the antigen itself (i.e., free of

association with the APC-binding domain) will not bind to the same APC or will bind at a significantly lower affinity (at least 10-fold lower).

The compositions of the present invention are distinguished from prior art molecules compositions comprising nonpeptide linkages between APC ligand and antigens in that the linkages of the invention can be produced using recombinant DNA techniques. Furthermore, this property allows an animal to be vaccinated with a nucleic acid encoding a fusion polypeptide of the invention, so that, when expressed as a secreted molecule *in vivo*, the fusion polypeptide is targeted to an APC, regardless of whether a non APC, e.g., a rhabdomyocyte, expressed it. This is important since, for example, after intramuscular nucleic acid injection a significant amount of the nucleic acid is taken up by rhabdomyocytes, which are not generally considered to be APCs, or are considered to be poor apcs.

In addition, the compositions of the invention are distinguished from fusion polypeptides comprising antibody idiotypes by having an APC BINDING portion that is a constituent of the innate immune system, i.e., that is substantially invariant among individuals of a species. Antibody idiotypes, in contrast, are generated and diversified in part by ontogenic processes such as VDJ recombination, junctional diversity, and somatic mutation. Thus, they vary substantially among individuals, and can be unique to individuals. Administration of both an alloidiotype and another polypeptide, therefore, will often constitute coadministration of at least two foreign antigens. Coadministration of two foreign antigens can result in "antigenic competition" (Hunt et al., *Vaccine* 12:45764; Rizvi et al., *Int J Exp Path* 71:76170; Hammerl et al., *Mol Immunol.* 25:31320; Johansson et al., *PNAS* 84:686973; Babbitt et al., *PNAS* 83:450913), so that the immune response to a fusion polypeptide comprising two foreign antigens, one of which is an idiotype, might be substantially different from the immune response to a polypeptide comprising one foreign antigen and a nonforeign opsonin. In addition, the polypeptides of the invention, unlike immunoglobulins with idiotypes that bind to molecules on the surface of APCs, can bind to antigens that are not expressed on the cell surface.

Opsonins Useful According to the Invention

The term "opsonin" is used herein interchangeably with "innate opsonin". Both terms refer to a naturally occurring secreted polypeptide molecule which in nature may become bound to an antigen and also bound to the surface of an APC. Furthermore, in nature the opsonin can be bound contemporaneously to both the antigen and the APC to form a complex that facilitates uptake of the

antigen by the APC.

Opsonins particularly useful in the invention are those which bind APCs of monocytic lineage. Monocyte-lineage APCs include, for example, monocytes, macrophages, and dendritic cells.

One particularly useful opsonin is a biologically active fragment of C3 and the APCs are uncultivated peripheral blood monocytes.

If the molecule comprises a fragment of C3, the APC binding domain must bind to CR1 with a greater affinity than it binds to CR2. This definition of a fragment of C3 therefore excludes C3d and C3bi.

An innate opsonin can bind to an antigen other than a molecule that is physiologically expressed on the surface of an APC.

According to the invention, innate opsonins are present in most individuals of a given species, and are structurally invariant among most members of a species, except that allelic variations may exist. During the ontogeny of most individuals, a gene encoding an innate opsonin does not undergo mutation or rearrangement in most of the cells that express the opsonin.

An opsonin can also be a polypeptide molecule, e.g., C3, which can be proteolytically processed such that at least one product of the processing step or steps can be bound stably and contemporaneously to an antigen, via a physiologically occurring linkage, and to the surface of an APC.

Other particularly useful opsonins bind to receptors on monocytelineage APCs such as receptors which play a role in innate immunity. Examples of such receptors include CR1, CR3, the C1q receptors and receptors containing a component of the C1q receptors. Examples of opsonins which can be used in the compositions and methods of the invention include fibronectin (e.g., Genbank accessions X02761, K00799, K02273, X82402, X00307, X00739), CRP (e.g., Genbank accessions X17496, M11880, M11881, M11882), complement components such as C1q (e.g., Genbank accessions X66295, M22531, X03084, X58861, and SwissProt accessions P02747, P02745), complement fragments such as C3b (e.g., Genbank accessions K02782, K02765), mannose binding protein (e.g., Genbank accessions S42292, S42294, X15422), conglutinin (e.g., Genbank accession X71774), alpha2macroglobulin (e.g., Genbank accessions M93264, M11313), and surfactant proteins A (e.g., Genbank accessions M68519, S48768) and D (e.g., Genbank accessions L40156, X65018, S38981), and their homologues among species.

There are a number of examples of opsonin fragments that comprise APC binding moieties. For example, Las Holtet et al., 1994, *FEBS Lett* 344:242 describe a carboxy-terminal fragment of human a2m (val1299-ala1451) that binds with high affinity to the a2m receptor. Fragments comprising amino acids 1314-1451 of human a2m and the corresponding domain of rat a2m also bind to a2m receptors, albeit with 1-2% of the affinities of native a2m (Van Leuven et al., 1986, *J Biol Chem* 261:11369; Enghild et al., 1989, *Biochemistry* 28:1406; Salvesen et al., 1992, *FEBS Lett* 313:198; Sottrup-Jensen et al., 1986, *FEBS Lett* 205:20).

Becherer and Lambris, 1988, *J Biol Chem* 263:14586 describe fragments of C3b that bind to CR1, e.g., C3c, fragments of C3 generated by elastase treatment and comprising the N-terminal of the alpha' chain of C3b, and a synthetic peptide comprising the 42 N-terminal amino acids of the C3b alpha' chain. A binding sequence in C3 for CR3 has also been described (Wright et al., 1987, *PNAS* 84:4235).

"Collagen stalks" of C1q, which are N-terminal fragments obtained by pepsin digestion, bind to the C1q receptor (Reid, 1981, *Methods Enzymol* 80:16; Malhotra et al., 1993, *Biochem J* 293:15). Malhotra et al., ibid., also provide evidence that an APC binding moiety of conglutinin is comprised by its 55 N-terminal amino acids. Ezekowitz (US Pat 5, 270, 199) offers a putative APC binding site in human mannose binding protein consisting of nucleotides 370-438 of Fig. 2 in the '199 Patent.

Families of Opsonins Useful According to the Invention

Some sets of opsonins can be regarded as structurally and functionally similar. For example, one family comprises fragments of complement components C3 and C4. These two components are highly structurally homologous, and each possesses an intramolecular thiolester bond that is broken when a peptide (C3a or C4a respectively) is proteolytically cleaved from the native molecule. Disruption of the thiolester makes available a chemical structure that can form an ester linkage with an antigen. The moiety of C3 on which this ester bond resides, i.e., the nonC3a moiety, is designated C3b, and C4b is the analogous product of C4 cleavage. C3b can be further proteolysed by proteins such as factor I to yield fragments such as C3bi and C3d, which also remain linked to the antigen via the ester bond.

However, not all biologically active fragments of C3 are opsonins according to the invention. For example, C3d does not bind to surface receptors on peripheral blood monocytes (refs). Its primary biological activity is thought to be to provide costimulatory transmembrane signals directly

to B lymphocytes through CR2. Furthermore, such an approach is limited to increasing a humoral immune response, whereas targeting antigens to monocytes can modulate either humoral or cellular immune responses, since APCs of monocytic lineage influence both types of response through their interactions with "helper" T cells.

There are four structurally unique proteins that are known to function as high affinity receptors for biologically active, membranebound fragments of C3 and/or C4. CR1 is the major receptor for the C3b fragment of C3 and C4b fragment of C4. It is expressed on monocytes and monocytederived APCs, among other cell types. CR2 is the major receptor for the fragment of C3 known as C3d, and is expressed on, e.g., mature B lymphocytes, but not on cells of monocytic lineage. The major role of CR2 on B lymphocytes is believed to be direct costimulation of B cells in concert with their cognate antigens.

CR3 is expressed primarily by neutrophils and monocytes and is also expressed on FDC, Kupffer cells, and NK cells. CR3 is a C3 fragment receptor with a primary specificity for C3bi. CR3 has been proposed as an important organizer of cytoskeletal events necessary for adhesive interactions and membrane reorganization during processes such as phagocytosis.

CR4 is a member of the beta2 integrin family, and its alpha chain is structurally similar to the alpha chain of CR3 and LFA1. Its primary physiologic ligand is believed to be C3d,g;, however, its biologic activities are less well understood than CR3.

Another example of a family of innate opsonins is the collectins, a group of collagenous Ctype lectins that comprises complement component C1q, mannose binding protein, surfactant proteins A and D, and conglutinin. Each molecule comprises a lectin domain that can bind to an antigen, and a collagenous domain that can bind to receptors on phagocytic mononuclear cells, including receptors that are wholly or partially identical to the C1q receptor (Tenner et al., *Immunity* 3:48593; Guan et al., *J Immunol.* 152:400516; Geertsma et al., *Am J Physiol* 267:L57884; Miyamura et al., *Biochem J* 300:23742; Malhotra et al., *J Exp Med* 172:9559; Malhotra et al., *Biochem J* 293:1519). Most known collectins comprise multiple polypeptide chains, in some cases homomeric and in others heteromeric, that are assembled posttranslationally, in part by covalent crosslinkage of hydroxyproline and hydroxylysine residues. Collectins are demonstrated to be opsonins in, for example, Pikaar et al., *J Infect Dis* 172:4819; AlvarezDominguez et al., *Infection & Immunity* 61:366472; O'Riordan et al., *J Clin Invest* 95:2699710; Kuhlman et al., *J Exp Med* 169:173345; and Geertsma et al., op cit.

Among the other innate opsonins useful according to the invention are Creactive protein

(CRP), alpha2 macroglobulin (a2m), and fibronectin. CRP, a member of the pentraxin family of molecules, binds to receptors on cells of monocytic lineage and has been shown to be an opsonin (Culley et al., *J Immunol.*, 1995, 156;4691). Alpha2 macroglobulin, like C3 and C4, comprises an internal thiolester bond that can be disrupted when the molecule is proteolysed. Such disruption allows covalent binding of the molecule to an antigen, and binding of alpha2 macroglobulin to an APC can promote uptake of the conjugate (Straight et al., *Biochemistry* 27:288590). Fibronectin binds to the alpha 5 beta 1 integrin and can also bind to various antigens, allowing it to function as an opsonin (Cosio, *J Lab Clin Med* 103:6139; Czop and Austen, *J Immunol.* 129:267881).

Fusion polypeptides have previously been constructed between portions of opsonins and relatively limited group of other polypeptides for purposes such as to facilitate isolation and purification of the opsonin or to elucidate structure function relationships. For example, a portion of alpha2 macroglobulin has been fused to carbonic anhydrase II to facilitate expression of the a2m moiety in a bacterium (MottaquiTabar et al., *Ann NY Acad Sci* 737 4935). Short portions of C3 from nonhuman species have been fused with human C3 to elucidate structure function relationships (Lambris et al., *J Immunol.* 156:482132). A heptapeptide recognition site for Tobacco Etch Virus (TEV) protease was introduced into alpha2 macroglobulin to investigate the function of the a2m bait region (Van Rompaey et al., *Biochem J* 312:1915). Carbohydrate recognition domains of opsonins or adhesion molecules have been fused to portions of opsonins in order to demonstrate transfer of ligand specificity (Blanck et al., *J Biol Chem* 271:728992; Ogasawara et al., *J Biol Chem* 269:2978592). Peptides comprising the ArgGlyAsp (RGD) APC BINDING moiety of fibronectin have been fused to a number of polypeptides in order to study, e.g., structure function relationships and amphibian cell biology (Alfandari et al., *Mech Dev* 56:8392; Ramos et al., *J Cell Biol* 134:22740; Ebeling et al., *Eur J Immunol.* 26:250816). The invention, in contrast, provides compositions that are useful for preventing or treating disease.

Opsonins are thought to act as a link or coupling agent between the antigen and the APC to allow more efficient binding, engulfment, and internalization of the antigen. A molecule is defined herein as an opsonin useful in the invention if it binds to a cognate antigen as determined in one or more of the assays of opsonicity described herein. According to the invention, opsonicity is determined in part by detection of binding to an APC and an antigen. For example, fragments of C3 can be bound to sheep red blood cells (SBRC); and opsonins with lectin activity can be directly admixed with microorganisms bearing a cognate carbohydrate.

According to the invention, an "APC binding domain" is a portion of an opsonin which

permits binding of a fusion polypeptide containing that domain and an antigen to an APC. A fusion polypeptide or a complex of the invention comprises an APC binding domain if it can bind to a naturally occurring APC surface molecule with an affinity at least in the nanomolar range and if binding to the molecules does not occur via the antigen. Binding via the antigen is easily discernible by testing free antigen for affinity to the surface molecule. Binding via antigen does not occur if the affinity of free antigen is at least 10-fold lower than that of the polypeptide or complex.

A "fusion polypeptide complex" contains first and second portions of an opsonin that together form an APC binding domain and together permit binding of a fusion polypeptide containing one such portion fused in-frame to an antigen to bind to an APC. A first or second portion of an APC binding domain does not correspond to an APC binding domain in itself, but forms an APC binding domain only when covalently associated with the second or first portion, respectively. This complex can be produced when the gene encoding the fusion polypeptide is expressed in a cell and coexpressed with a gene encoding a chain of an opsonin that contains the other portion (first or second portion) of the APC binding domain.

An APC binding domain will also, of course, includes a complete opsonin polypeptide, e.g., C3.

It is particularly preferred according to the invention where the APC binding domain consists essentially of an APC BINDING moiety of an innate opsonin.

A fusion polypeptide according to the invention comprises an "APC binding domain of an opsonin" if the fusion polypeptide can bind to a receptor that is physiologically expressed on an APC with an affinity at least in the nanomolar range. Fusion polypeptides according to the invention do not include chimeric proteins consisting only of a first opsonin or APC binding domain thereof fused to a second different opsonin, or APC binding domain thereof, but may include one or more opsonins or APC binding domains thereof fused to an antigen.

A fusion polypeptide or multichain complex of the invention will bind to the antigen presenting cell via the opsonin portion of the molecule rather than via the antigen. This is easily distinguishable as free antigen will not compete with a fusion polypeptide for APC binding if the polypeptide or complex binds to the APC via the APC binding domain, whereas free antigen will compete with the fusion polypeptide binding to the APC if the polypeptide or complex binds to the APC via the antigen portion of the polypeptide or complex. Therefore, a fusion polypeptide of the invention comprises an APC binding domain of an opsonin if this APC binding domain can bind to a receptor that is physiologically expressed on an APC with an affinity at least in the nanomolar

range when included in a fusion polypeptide that does not comprise a second portion, heterologous to the first opsonin, which, in isolation, can bind to a receptor that is physiologically expressed on an APC with an affinity at least in the nanomolar range. APC binding domains that do not comprise entire opsonins have been described, for example, for mannose binding protein (Tenner et al., *Immunity* 3:48595), C3b (Becherer and Lambris, *J Biol Chem* 263:145891), conglutinin (Malhotra et al., *Biochem J* 293:1519), and fibronectin (Czop and Austen, *J Immunol.* 129:267881).

In another preferred embodiment, the APC binding moiety does not require the amino acid sequence RGD in order to bind to an APC receptor.

Assays for Determining Opsonicity According to the Invention

Assay 1

In one assay of opsonicity, as described by O'Rear and Ross in Current Protocols in *Immunology*, 1994, John Wiley & Sons, pp. 13.4.59, SRBC bound via a physiologically occurring linkage to the candidate opsonin molecule are obtained. APCs from the species to which the candidate opsonin is native are suspended at $4x10^6$/ml in ice-cold HBSS with 1% (w/v) Cohn fraction of BSA. If the candidate opsonin is a fragment of C3, the APCs are freshly drawn, uncultivated peripheral blood monocytes. SRBC linked to the candidate opsonin or control SRBC (identical to the former but not linked to the candidate opsonin) are suspended in the same solution at $2x10^8$/ml. 100ul of SRBC suspension and 100ul of APC suspension are mixed in a 10 x 75 mm plastic tube. The tube is rotated at 40 rpm at 37°C for 2 20 min. A small drop of the suspension is placed on a slide, covered with a coverslip, and allowed to stand for 510 min. Excess fluid can be removed by pressure on the coverslip, and the coverslip can be sealed to the slide, e.g., with clear nail polish. The slide is examined microscopically, and the percentage of APCs visibly adherent to 4 or more SRBCs is determined. If the percentage is 50% or greater when there are up to $4x10^4$ candidate opsonin molecules/SRBC', the candidate opsonin can be an opsonin.

Assay 2 (For protease-activated candidate opsonin)

Candidate opsonin or radiolabeled Candidate opsonin is treated with a 1.5-3 fold molar excess of protease (0.05 M triethanolamine-0.1 M NaCl, pH 8.0, room temperature overnight). In this assay, the protease can serve as the antigen or an excess of another antigen can be added. Prior to binding studies, the candidate opsonin-antigen complex is dialyzed against HBSS (4°C).

Candidate opsonin -antigen complex binding to monocytes is measured by incubating labeled ligand at a concentration up to 1.0 M with (1.5-4.0) x 10^6 monocytes in 200 ml volume on ice.

Nonspecific binding of radiolabeled ligands is determined in the presence of a 100-fold molar excess labeled candidate opsonin-antigen complex. The unbound ligand is separated from the cells and cell-bound ligand by rapid vacuum filtration on glass fiber filters. Studies are performed on ice to avoid potential complications due to endocytosis. Binding constarts and the number of sites per cell are determined by analysis and by nonlinear curve fit. If candidate opsonin-antigen complex affinity for a monocyte binding site is in at least the nanomolar range, the candidate opsonin is an opsonin.

Assay 3

Part I

To directly evaluate whether candidate opsonin is bound to the surface of *P. carinii*, immunoelectron microscopy is performed. *P. carinii* are isolated from bronchoaveolar lavage (BAL) of moribund infected rats using TBS with 1 mM calcium to preserve surface-bound candidate opsonin. Isolated organisms are fixed in periodate-lysine-paraformaldehyde buffer and embedded in Lowacryl mounting medium (Ted Pella, Inc., Redding, Calif.). Ultrathin sections are obtained, blocked with normal goat serum (2%) for 1 h, and incubated with either rabbit anti-candidate opsonin or nonimmune rabbit IgG (25 mg/ml) overnight. After washing, the sections are subsequently incubated with goat and rabbit IgG conjugated to 15 nM colloidal gold (Amersham Corp., Arlington Heights, IL). The sections are washed again and examined on a transmission electron microscope (model 6400:JEOL USA, Inc., Peabody, MA).

Part II

The attachment of *P. carinii* to cultured alveolar macrophages in the presence or absence of antibody to SP-D or with the addition of purified SP-D is quantified as follows. Adherence of *P. carinii* to alveolar macrophages is assayed by ^{51}Cr-labeling the organisms. *P. carinii* are isolated from infected rats with TBS containing 1 mM calcium to prevent loss of surface-bound candidate opsonin. The organisms are radiolabeled by incubation for 8 h at 37°C in 2 ml of DME containing 20% FCS and 200 μCi of ^{51}Cr-sodium chromate (New England Nuclear). Normal alveolar macrophages are lavaged from healthy rats and plated in tissue culture plates (1 X 10^5) cells/well) which are been precoated with normal rat IgG (100 mg/ml X 60 min) in order to ensure firm adherence of the macrophages. After 1 h, the macrophages are gently washed with HBSS to remove nonadherent cells. >95% of macrophages are adherent after this wash. ^{51}Cr-*P. carinii* (1 X 10.) containing surface-associated candidate opsonin are added to the macrophages and incubated at 37°C

for an additional hour. Subsequently, nonadherent *P. carinii* are removed by washing. The macrophage monolayers containing adherent *P. carinii* are solubilized in 1 N NaOH and quantified. Adherence of *P. carinii* is defined as: percentage of adherence = (A/A+ B) X 100, where A = ^{51}Cr-*P. carinii* associated with the monolayer, and B = unattached ^{51}Cr-*P. carinii*. To assess the effect of candidate opsonin on the attachment of *P. carinii* to alveolar macrophage lung cells in culture, *P. carinii* adherence assays are conducted in the presence or absence of a polyclonal rabbit antibody generated against the candidate opsonin (100 mg/ml).

If candidate opsonin binding to *P. carinii* is apparent in Part I and if, in Part II, % adherence is diminished in the presence of anti-candidate opsonin with statistical significance of $P<0.05$, the candidate opsonin is an opsonin.

Assay 4

Association of bacteria with adherent monocytes is measured as follows. Endotoxin level in the modified PBS and in all buffers used is below 50 pg/ml as determined by the Limulus assay. 5 x 10^3 monocytes in modified PBS are allowed to adhere to the wells of a Terasaki plate for 2 h at 37°C. After nonadherent cells are removed by three washes with PBS, 5 X 10^4 FITC-labeled bacteria in 0.5 ml buffer with or without 10-50 micrograms/ml of candidate opsonin are added. A bacteria-to-monocyte ratio of 10:1 to 50:1 is used. After 30 min of incubation at 37°C in the dark, the nonadherent bacteria are removed by five washes with warm PBS. Assays are performed in quadruplicate; in each well, the number of bacteria associated with ^{3} 100 monocytes is counted under a florescence microscope using x 400 magnification. Results are expressed as the number of bacteria associated with 100 monocytes. If this number with candidate opsonin can be at least twice that without candidate opsonin, the candidate opsonin is an opsonin.

Assay 5

Part I

About 1 x 10^7 to 6 x 10^7 bacteria per ml are incubated (20 min, 0°C) with 10 mcg/ml of ^{125}I-candidate opsonin in a total volume of 0.7 ml. of PBS aliquots, 100 ml, of the reaction mixtures are layered over 150 ml of an oil cushion (60% dibutyl phthalate, 40% dioctyl phthalate [Eastman Kodak Co., Rochester, N.Y.]), and the mixtures are centrifuged (10,000 x g, 60 s, 4°C). The tip of the tube, containing the cell pellet, is cut with a Mozart razor blade, and the radioactivity is counted.

Part II

APCs are plated in 96-well tissue culture plates (Costar, Cambridge, Mass.) at 2 x 10^5 cells per ml the evening before use. 2 x 10^6 bacteria per well (0.1 ml per well) are added to the culture plates with or without 100 mcg/ml of candidate opsonin. The plates are then centrifuged at 1,000 x g for 7 min. After 15 min at 37°C to allow the uptake of bacteria, free bacteria are removed by several washes with cold PBS. They are then incubated (45 min, 37°C) in RPMI 1640 plus an amount of antibiotic that, when present in the culture for 45 min, kills all extracellular bacteria. The end of this incubation period is considered time zero. Monolayers are washed three times with Hanks' balanced saline solution, and the same volume of RPMI 1640 (R0) is added. The cells are lysed by using several cycles of freezing and thawing. The number (CFU) of viable bacteria per well is determined by quantitative plate counts on blood agar plates (Columbia blood agar; Becton Dickinson, San Jose, Calif.) after 24 h of incubation. Each result is given as the mean of three determinations.

If, in Part I, candidate opsonin-treated bacterial pellet has >75 KCPM and this incorporation can be inhibited by unlabeled candidate opsonin, and if in Part II the CFU with candidate opsonin is greater than without ($P< 0.05$), the candidate opsonin can be an opsonin.

Assay 6

200 μl of GHBSS (Hanks Balanced Salt Solution) +0.1%of gelatin containing 10 m mol $CaCl_2$) containing 10^7 bacteria is prepared. The bacteria are then incubated at 4°C with 20-100 μg/ml of candidate opsonin. Binding assays are done in the presence or absence of a competitive inhibitor. After incubation for 30 minutes, the bacteria are washed five times in a GHBSS + 10 mmol $CaCl_2$ at room temperature in a microfuge at 1,300 g for 3 minutes. Thereafter, a 1:1,000 dilution of rabbit anti-candidate opsonin antiserum is incubated with the bacteria for 1 h in PBS + 5% FCS and 10 mmol $CaCl_2$ and then the bacteria are washed three times in GHBSS + 10 mmol $CaCl_2$ plus 0.05% Tween 20. Binding of anti-serum to bacteria is detected by a 1:1,000 dilution of goat anti-rabbit IgG conjugated to rhodamine (Fisher Pharmaceuticals, Orangeburg, NY). After incubation, the bacteria are washed five times in GHBSS + 10 mmol $CaCl_2$ plus 0.05% Tween 20, smeared onto glass slides and allowed to air dry. Thereafter bacteria are fixed with 100% ice cold methanol for 5 minutes. Negative controls included the absence of candidate opsonin and no first step antibody. Numerous fields of triplicate assays are examined by fluorescence microscopy.

Part II *Association of Radiolabeled Bacteria with Cells.*

10^7 radiolabeled bacteria are resuspended in 200 μl of GHBSS + 10 mmol $CaCl_2$ and are

incubated with or without candidate opsonin ranging from 2 μg/ml to 40 μg/ml at 4°C for 30 min. The bacteria are then washed three times in GHBSS + 10 mmol $CaCl_2$ for 3 min at room temperature in a microfuge at 1,300 g, resuspended in 50 μl of GHBSS and added to a 1-ml suspension containing on the order of 10^6 APCs (GHBSS). The bacteria and APCs are gently rocked at 37°C for 20 min and thereafter the unattached bacteria are removed by five washes using differential centrifugation at 82 g in a microfuge. Before the last wash, an aliquot from each sample is plated on a Labtek slide and cells are adhered for 10 min, fixed in methanol, stained with Geimsa, and scored by light microscopy. To score the cells plated on the Labtek slides, at least 400 cells are counted. The phagocytic index represented the number of attached or ingested particles per 100 PMNs. The pellet from above containing cells and radiolabeled bacteria is then lysed in 100 μl PBS + 0.5% Triton X-100 and the radioactivity is measured in a scintillation counter. If, in Part I, specific binding of candidate opsonin to bacteria is evident, and in Part II the specific uptake of bacteria, in cpm, is more than three times greater with candidate opsonin than without, the candidate opsonin can be an opsonin.

Assay 7

Part I

To investigate binding to *L donovani promastigotes* cultures are seeded at 5 x 10^5 parasites ml^{-1}. At regular time points up to 9 days, a fraction of parasites are counted, washed, and resuspended in 1% BSA, 0.5 mM Ca^{2+}. 0.05% NaN_3, Tris-buffered saline (TBS), (10 mM Tris-HCl, 0.15 M NaCl, pH 8.0) (diluent) to 2 x 10^5 ml^{-1}. Fifty microliters of this suspension are then added to 200-μl microfuge tubes containing 70 μl 5 μg/ml radiolabled C-reactive protein (CRP) (0.12 μCi/μg) in diluent without EDTA, which had been layered over 150 μl of a dinonyl phthalate/dibutyl phthalate (40:60 v/v) oil mixture. Parasites are incubated for 1 h and centrifuged through the oil layer, the cell pellet Is cut off, and associated CRP is detected by gamma counting. Each assay is performed in triplicate. The concentration dependency of CRP binding to promastigotes is also measured as above, using an activity of 0.045 μCi/μg and a twofold dilution series from 60 to 0.015 μg/ml CRP.

Part II

APCs are plated out at 1 x 10^6 cells/well on glass coverslips in a 24-well tissue culture plate. Cells are incubated in RPMI 1640 (Life Technologies) supplemented with 10% PCS, 1 mM glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin in a humidified incubator at 37°C. After

24 h, nonadherent cells are removed and remaining cells are used after 6 days. Promastigotes are incubated with or without CRP at 30 μg/ml in RPMI 1640 for 1 h and then washed three times before adding to the APC cultures at 10^6/well. Promastigotes are allowed to infect APCs for 1 h, then cells are washed, fixed with methanol, and Geimsa stained (BDH, Poole, Dorset, U.K.) before counting. The percentage of APCs infected and the number of parasites/100 macrophages is determined from quadruplicate cultures.

If in Part I the affinity of candidate opsonin for parasites is at least in the nanomolar range and in Part II the number of parasites taken up/100 APCs is, with candidate opsonin, at least twice that without candidate opsonin, the candidate opsonin can be an opsonin.

Assay 8

Part I

Portions (0.5 ml) of [^{35}S]methionine-labeled culture medium containing 5 percent fetal calf serum and the candidate opsonin are incubated for 30 minutes at room temperature with 0.1 ml or 0.2 ml of a 10 percent suspension of a microorganism). The microorganisms tested may include, for example, *Salmonella typhimurium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*. Bound proteins are released by boiling in buffer containing 2 percent SDS and 0.1 M dithiothreitol and are analyzed on a 5 percent SDS gel.

Part II

Fixed bacteria (0.1 ml; 10 percent by volume; 10^{10} organisms per milliliter), labeled with [^{3}H]thymidine, are incubated with 0.1 ml of serum with or without depletion of the candidate opsonin. After being washed with PBS, the bacteria are incubated with on the order of 1 x 10^7 APCs in a final volume of 0.9 ml PBS containing divalent cations. At intervals 0.2 ml is removed to ice-cold PBS with N-ethyimaleimide (2mM) to block further endocytosis, and the cells are washed (at about 100g for 10 seconds).

If in Part I a band corresponding to the candidate opsonin is apparent, and if in Part II the CPM after 6-10 min of incubation is at least three times greater for undepleted samples with serum than with depleted serum, the candidate opsonin can be an opsonin.

In lieu of results form Parts I of assays 3, 5, 6, 7, 8, a candidate opsonin that satisfies Part II of an assay can be an opsonin if it can bind to the antigen of the assay with an affinity in at least the nanomolar range.

Linkage of Antigen to Ligand

An antigen is linked to ligand, e.g., an opsonin, according to the invention via recombinant DNA techniques to form a chimeric gene, and expression of the chimeric gene in a host cell. Therefore, the linkage contemplated in the invention is limited to a peptide linkage for formation of an in-frame fusion polypeptide.

A flexible linker sequence may be inserted into the fusion polypeptide between the antigen and the ligand. For example, a polygylcine/polyserine-containing sequence such as $(Gly_4Ser)_2$. See Huston et al., 1991, *Meth. Enzymol.* 203:46.

Production of Fusion Polypeptide and Fusion Polypeptide Complexes

A fusion polypeptide according to the invention is produced *in vivo* or in vitro in a host cell by expression of the gene encoding the fusion polypeptide or by coexpression of the gene encoding the fusion polypeptide and a gene encoding a polypeptide chain which when associated with the fusion polypeptide to form a complex, permits binding of the complex to a cell, e.g., an APC. When two such genes are coexpressed, the fusion polypeptide and the polypeptide chain naturally form an association in the cell, for example, by disulfide bonding or by cross-linking between hydroxylated amino acids, such as via a lysine or proline residue.

Nucleic Acid Sequences

Another aspect of the invention features isolated nucleic acid molecules which include a nucleotide sequence encoding an antigen fused in one reading frame to a nucleotide sequence encoding a cell binding domain, e.g., an APC binding domain of an innate opsonin. The nucleic acid molecule also may include a sequence encoding a first or second portion of a ligand, which portions correspond to fragments of the ligand that form a cell binding domain. Where the nucleic acid sequence encodes a polypeptide complex according to the invention, the sequence encoding the fusion polypeptide and the sequence encoding the chain which in association with the fusion polypeptide forms an cell binding domain may be coexpressed and therefore under coordinated or the same gene control elements.

The phrase "nucleic acid molecule" as used herein is intended to include such fragments and refers to DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA). The nucleic acid molecule can be singlestranded or doublestranded, but preferably is doublestranded DNA. An "isolated" nucleic acid molecule is free of sequences which flank the nucleic acid (i.e.,

sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be free of other cellular material. The term "fragment" as used herein refers to a portion of a nucleic acid molecule or polypeptide or other molecule described herein.

Typically, the nucleotide sequence encoding the antigen and the nucleotide sequence encoding the cell binding domain can be fused to form a "fusion gene" according to techniques known in the art. For example, in one embodiment, attachment/linkage of nucleic acid fragments coding for different polypeptide sequences can be performed in accordance with conventional techniques, employing bluntended or staggerended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be performed using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (*see, e.g., Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

Cytokines Useful According to the Invention

The term "cytokine" as defined herein refers to a polypeptide molecule that is naturally secreted by mammalian cells and that binds to a cell surface receptor on a leukocyte. The term "cytokine" also refers herein to a polypeptide molecule that is a ligand for a receptor for a naturally occurring cytokine. Unlike an opsonin, a cytokine does not naturally contemporaneously bind an antigen and a cell-surface receptor.

Leukocytes which bear receptors for cytokines include, for example, monocytes, macrophages, dendritic cells, neutrophils, eosinophils, basophils, platelets, lymphocytes, T lymphocytes, B lymphocytes, NK cells, myeloma cells, lymphoma cells, and leukemic cells.

Preferred cytokines are non-rodent cytokines, e.g., primate, e.g., human cytokines.

Some cytokines can be regarded as belonging to one or more families of cytokines based on structural and/or functional properties. One such family consists of the interleukins. Interleukins are structurally diverse, but share the property of both being expressed by and acting on leukocytes. Examples of interleukins include IL-1 (e.g., polypeptides encoded by Genbank Accession No. M15330, M28983, E04743, M15131) IL-2 (e.g., polypeptides encoded by Genbank Accession No E01108, K02797), IL-3 (e.g., polypeptides encoded by Genbank Accession No A02046, M14743),

IL-4 (e.g., polypeptides encoded by Genbank Accession No M13982, M25892), IL-5 (e.g., polypeptides encoded by Genbank Accession No X06270, J03478), IL-6 (e.g., polypeptides encoded by Genbank Accession No E02772, M20572), IL-7 (e.g., polypeptides encoded by Genbank Accession No J04156, M29054-29057), IL-8 (e.g., polypeptides encoded by Genbank Accession No M28130), IL-9 (e.g., sequences disclosed in Kelleher et al, Blood. 1991; 77: 1436-1441, Immunogenetics 1990;31(4):265-270), IL-10 (e.g., polypeptides encoded by Genbank Accession No M84340, U16720), IL-11 (e.g., sequences disclosed in Paul et al, Proc Natl Acad Sci U S A. 1990; 87: 7512-7516, Morris et al, Exp Hematol. 1996; 24: 1369-1376), IL-12 (e.g., polypeptides encoded by Genbank Accession No. M86671, S82412; GENBANK PROTEIN P29459, P29460), IL-13 (e.g., polypeptides encoded by Genbank Accession No U31120, L13028), 1L-14 (e.g., sequences disclosed in Ambrus et al, Proceedings Of The National Academy Of Science (USA) 1993; 90: 6330-4), IL-15 (e.g., polypeptides encoded by Genbank Accession No AF031167, U22339), 1L-16 (e.g., polypeptides encoded by Genbank Accession No AF006001, M90391), IL-17 (e.g., polypeptides encoded by Genbank Accession No U32659, U43088), IL-18 (e.g., polypeptides encoded by Genbank Accession No D49949, D49950), TNF-alpha (e.g., polypeptides encoded by Genbank Accession No M16441, Y00467), and GM-CSF (e.g., polypeptides encoded by Genbank Accession No X03019, M11220) and their homologues among species. Nucleotide sequences encoding homologues will hybridize to each other under moderate- to high-stringency conditions.

Another family consists of the hematopoietins. members of this family comprise four alpha helical regions, known as helices A, B, C, and D. Helices A and B and helices C and D run roughly parallel to each other, respectively. Examples of hematopoietins include IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, 1L-15, GM-CSF, G-CSF (e.g., polypeptides encoded by Genbank Accession No. E01219, M13926), ONCOSTATIN M (e.g., polypeptides encoded by Genbank Accession No. D31942, sequences disclosed in Malik et al, *Mol Cell Biol* 1989;**9:**2847-2853), LIF (e.g., polypeptides encoded by Genbank Accession No. X13967, X06381), CNTF (e.g., polypeptides encoded by Genbank Accession No. U05342, X60542), and their homologues among species. Nucleotide sequences encoding homologues will hybridize to each other under moderate- to high-stringency conditions.

Human IL2 is a protein of 133 amino acids (15.4 kDa) with a slightly basic pI. Murine and human IL2 display a homology of approximately 65%. IL2 is synthesized as a precursor protein of 153 amino acids with the first 20 amino-terminal amino acids functioning as a hydrophobic secretory signal sequence. The protein contains a single disulfide bond (positions cys58/105) essential for

biological activity.

IL2 is O-glycosylated at threonine at position 3. Variants with different molecular masses and charges are due to variable glycosylation. Non-glycosylated IL2 is also biologically active. Glycosylation appears to promote elimination of the factor by hepatocytes.

A dimeric form of human IL2, produced by the action of a transglutaminase isolated from regenerating fish optic nerves, has been shown to be a cytotoxic factor for rat brain oligodendrocytes in culture.

The human IL2 gene contains four exons. The IL2 gene maps to human chromosome 4q26-28 (murine chromosome 3). The homology of murine and human IL2 is 72% at the nucleotide level in the coding region.

The biological activities of IL2 are mediated by a membrane receptor that is expressed almost exclusively on activated, but not on resting, T-cells at densities of $4\text{-}12\times10^3$ receptors/cell. Activated B-cells and resting mononuclear leukocytes rarely express this receptor. The expression of the IL2 receptor is modulated by IL5 and IL6. Three different types of IL2 receptors are distinguished that are expressed differentially and independently. The high affinity IL2 receptor (Kdis ~10 pM) constitutes approximately 10% of all IL2 receptors expressed by a cell. This receptor is a membrane receptor complex consisting of the two subunits IL2r-alpha (TAC antigen = T-cell activation antigen; p55) and IL2r-beta (p75; CD122) as the ligand binding domains and a gamma chain as a signaling component. p75 is expressed constitutively on resting T-lymphocytes, NK-cells, and a number of other cell types while the expression of p55 is usually observed only after cell activation. p55 is, however, synthesized constitutively by a number of tumor cells and by HTLV-1-infected cells.

IL2 receptor expression of monocytes is induced by IFN-gamma, so that these cells become tumor-cytotoxic. In T-cells the expression of p75 can be reduced by IL3.

An intermediate affinity IL2 receptor (Kdis =100 pM) consists of the p75 subunit and a gamma chain (see below) while a low affinity receptor (Kdis =10 nm) is formed by p55 alone.

p55 (e.g., polypeptides encoded by Genbank accession no. X01057) has a length of 251 amino acids with an extracellular domain of 219 amino acids and a very short cytoplasmic domain of 13 amino acids. The p55 gene maps to human chromosome 10p14-p15.

p75 (e.g., polypeptides encoded by Genbank accession no. M26062, m28052) has a length of 525 amino acids with an extracellular domain of 214 amino acids and a cytoplasmic domain of 286 amino acids. The p75 gene contains 10 exons and has a length of approximately 24 kb. It maps to human chromosome 22q11. 2-q12 and to murine chromosome 15 (band e).

A third 64 kDa subunit of the IL2 receptor, designated gamma, has been described (e.g., polypeptides encoded by Genbank accession no. D13821, d11086). Murine and human gamma subunits of the receptor have approximately 70% sequence identity at the nucleotide and amino acid levels. This subunit is required for the generation of high and intermediate affinity IL2 receptors but does not bind IL2 by itself. These two receptor types consist of an alpha-beta-gamma heterotrimer and a beta-gamma heterodimer, respectively. The gene encoding the gamma subunit of the IL2 receptor maps to human chromosome Xq13, spans approximately 4.2 kb and contains eight exons. The gamma subunit of the IL2 receptor has been shown recently to be a component of the receptors for IL4 and IL7. It is also believed to be a component of the IL13 receptor.

The amino acids at positions 267-317 lying directly adjacent to the transmembrane region of p75 are involved in IL2-mediated signal transduction. In addition the IL2 receptor is associated with a number of other proteins (p22, p40, p100) which are thought to be involved in mediating conformational changes in the receptor chains, receptor-mediated endocytosis, and further signal transduction processes. One of the identified proteins is the 95 kDa cell adhesion molecule ICAM-1 which probably focuses IL2 receptors at regions of cell-to-cell contacts and thus may mediate paracrine activities, for example, during IL2-mediated stimulation of T-cells. Another protein associated with p75 is a tyrosine-specific protein kinase called lck. The observation that proliferation of cells induced by IL2 is inhibited by specific inhibitors of protein tyrosine kinases in an lck negative cell line suggests that other kinases may also be associated with IL2 receptors. Two such kinases, called fyn and lyn, have been identified. In addition, IL2 receptor signaling may also be mediated by vav.

Activated lymphocytes continuously secrete a 42 kDa fragment of the TAC antigen. This fragment circulates in the serum and plasma and functions as a soluble IL2 receptor (sIL2r). The concentrations of this soluble receptor vary markedly in different pathological situations, for example, infections, autoimmune diseases, leukemias, or after organ transplantation. Levels may increase up to 100-fold. The levels of sIL2r appear to correlate with the severity of HIV-induced diseases and may be of diagnostic value also in other settings.

Mouse and human IL2 both cause proliferation of T-cells of the homologous species at high efficiency. Human IL2 also stimulates proliferation of mouse T-cells at similar concentrations, whereas mouse IL2 stimulates human T-cells at a lower (sixfold to 170-fold) efficiency.

Il2 is a growth factor for all subpopulations of T-lymphocytes. It is an antigen-unspecific proliferation factor for T-cells that induces cell cycle progression in resting cells and thus allows

clonal expansion of activated T-lymphocytes. This effect is modulated by hormones such as prolactin.

Il2 also promotes the proliferation of activated B-cells also this requires the presence of additional factors, for example, IL4.

Due to its effects on T-cells and B-cells IL2 is a central regulator of immune responses. It also plays a role in anti-inflammatory reactions, in hematopoiesis and in tumor surveillance. Il2 stimulates the synthesis of IFN-gamma in peripheral leukocytes and also induces the secretion of IL1, TNF-alpha and TNF-beta.

It is believed that he induction of the secretion of tumoricidal cytokines apart from the activity in the expansion of LAK cells (lymphokine-activated killer cells) are probably the main factors responsible for the antitumor activity of IL2.

Il2 can be assayed in bioassays employing cell lines that respond to the factor (e.g., ath8, ct6, ctll-2, fdcpmix, ht-2, nkc3, tall-103). Specific ELISA assays for IL2 and enzyme immunoassays for the soluble receptor are also available. The soluble receptor can be detected also by employing biotinylated IL2 and flow-through cytometry or ELISA assays.

Il2 displays significant anti-tumor activity for a variety of tumor cell types since it supports the proliferation and clonal expansion of T-cells that specifically attack certain tumors. Il2 is increasingly used to treat patients with cancers refractory to conventional treatment. Combination therapy with systemically administered IL2 has resulted in long-term remissions in 30% of patients with metastatic renal cell carcinoma, for which there is no standard treatment. Objective and long-lived clinical responses have been documented also in a proportion of patients with melanoma or acute myeloid leukemia.

High dose systemic IL2 therapy is also associated with a great number of unwanted toxic side-effects. Il2 has additional effects on other components of the cellular immune system, including B-cells and macrophages, and induces the secretion of other soluble mediators, including TNF-alpha, TNF-beta, and IFN-gamma. These effects may contribute to the antitumor activity of IL2 as well as to its dose-related toxicity.

The transduction of murine tumor cells with a functional IL2 gene has been shown to lead to the rejection of the genetically modified cells by syngeneic hosts. Altered tumor cells expressing IL2 also increase systemic immunity.

Human IL4 is a protein of 129 amino acids (20 kDa) that is synthesized as a precursor containing a hydrophobic secretory signal sequence of 24 amino acids. Il4 is glycosylated at two arginine residues (positions 38 and 105) and contains six cysteine residues involved in disulfide

bond formation. The disulfide bonds are essential for biological activity. Some glycosylation variants of IL4 have been described that differ in their biological activities. A comparison of murine and human IL4 shows that both proteins only diverge at positions 91-128.

An IL4 variant, y124d, in which tyr124 of the recombinant human protein is substituted by an aspartic acid residue, binds with high affinity to the IL4 receptor (Kd =310 pM). This variant is a powerful antagonist for the IL4 receptor system. It retains no detectable proliferative activity for T-cells and competitively inhibits IL4-dependent T-cell proliferation (K(i) = 620 pM). The existence of this mutant demonstrates that high affinity binding and signal generation can be uncoupled efficiently in a ligand. Y124d also acts as a powerful antagonist for the IL13 receptor.

The human IL4 gene contains four exons and has a length of approximately 10 kb. It maps to chromosome 5q23-31. The murine gene maps to chromosome 11. The IL4 gene is in close proximity to other genes encoding hematopoietic growth factors (e.g., GM-CSF, M-CSF, IL3, IL5). The distance between the IL4 and the IL5 gene is approximately 90-240 kb.

At the nucleotide level the human and the murine IL4 gene display approximately 70% homology. The 5' region of the IL4 contains several sequence elements, designated CLE (conserved lymphokine element), that are binding sites for transcription factors controlling the expression of this and other genes. A sequence motif, called p sequence (cgaaaatttcc) in the 5' region of the human IL4 gene (positions -79 - - 69) is the binding site for a nuclear factor, called NF(p), mediating the response to T-cell activation signals.

The biological activities of IL4 are mediated by a specific receptor (Kdis =20-100 pM) which is expressed at densities of 100-5000 copies/cell (e.g., polypeptides encoded by Genbank accession no. M29854, x52425). The extracellular domain of the IL4 receptor is related to the receptors for erythropoietin (epo), IL6, and the beta chain of the IL2 receptor. It has been given the name CD124.

The cDNA for the murine IL4 receptor encodes a transmembrane protein of 810 amino acids (including a secretory signal sequence). This receptor has a large intracellular domain of 553 amino acids. The human receptor has an extracellular domain of 207 amino acids, a transmembrane domain of 24 residues, and a large intracellular domain of 569 amino acids.

The IL4 receptor has been shown recently to contain the gamma subunit of the IL2 receptor as a signaling component. This gamma subunit is also associated with the receptors for IL4 and IL7 and probably also of IL13. Two forms of the receptor have been described, one of which is secreted. The secreted receptor only contains the extracellular IL4 binding domain and is capable of blocking IL4 activities. An IL4 binding protein (IL4-bp) that binds IL4 with the same affinity as the IL4 receptor has been shown also to be a soluble IL4 receptor variant. These soluble receptors probably

function as physiological regulators of cytokine activities by inhibiting receptor binding or act as transport proteins. Soluble receptors or binding proteins have been described also for IL1 (IL1 receptor antagonist), IL2, IL6, IL7, TNF-alpha, IGF, and IFN-gamma.

The biological activities of IL4 are species-specific; mouse IL4 is inactive on human cells and human IL4 is inactive on murine cells. IL4 promotes the proliferation and differentiation of activated B-cells, the expression of class II MHC antigens, and of low-affinity IgE receptors in resting B-cells. IL4 enhances expression of class II MHC antigens on B-cells. It can promote their capacity to respond to other B-cell stimuli. This may be one way to promote the clonal expansion of specific B-cells and the immune system may thus be able to respond to very low concentrations of antigens. The production of IL4 by non-B non-T-cells is stimulated if these cells interact with other cells via their Fc receptors for IgE or IgG. This effect can be enhanced by IL3. IL2 and PAF (platelet activating factor) induce the synthesis of IL4 while TGF-beta inhibits it.

IL3 antagonizes theIL2-induced effects in B-cells and causes a slow decrease of the expression of IL2 receptors, thus inhibiting the proliferation of human B-cells stimulated by IL2. In activated B-cells IL4 stimulates the synthesis of IgG1 and IgE and inhibits the synthesis of IgM, IgG3, IgG2a and IgG2b. This isotype switching induced by IL4 in B-cells is antagonized by IFN-gamma. The growth of multiple myelomas can be suppressed by IL4 which inhibits the synthesis of IL6, a myeloma growth factor. IL4 also inhibits the synthesis of IL6 in human alveolar macrophages.

Pretreatment of macrophages with IL4 prevents the production of IL1, TNF-alpha and prostaglandins in response to activation of the cells by bacterial endotoxins or IFN-gamma.

IL4 synergises with epo and G-CSF/epo in the generation of colonies containing granulocytes or erythroid progenitor cells in a colony formation assay.

The classical detection method for IL4 is a B-cell costimulation assay measuring the enhanced proliferation of stimulated purified B-cells. IL4 can be detected also in bioassays, employing IL4-responsive cells (e.g., balm-4; bcl1; ct.4s; ctl44; ctll-2; da; fdcpmix; ht-2; l4; l138.8a; mo7e; mc/9; nfs-60; ramos, sez627, tf-1; ts1). A specific detection method for human IL4 is the induction of CD23 in a number of B-cell lines with CD23 detected either by flow-through cytometry or by a fluorescence immunoassay. An immunoassay that allows rapid determination of the rate of IL4 production under conditions preventing consumption/degradation is cytokine immunotrapping.

IL4 inhibits the growth of colon and mammary carcinomas. It has been shown to augment the development of LAK cells. The transduction of murine tumor cells with a functional IL4 gene has been shown to lead to the rejection of the genetically modified cells by syngeneic hosts. Altered

tumor cells expressing IL4 also increase systemic immunity. Mice vaccinated with transduced cells reject a subsequent challenge of non-transduced cells, and, in some cases, a pre-existing tumor.

Human IL6 is a protein of 185 amino acids glycosylated at positions 73 and 172. It is synthesized as a precursor protein of 212 amino acids. Monocytes express at least five different molecular forms of IL6 with molecular masses of 21.5-28 kDa. They mainly differ by post-translational alterations such as glycosylation and phosphorylation.

IL6 isolated from various cell types shows some microheterogeneity in its N-terminus. A 42-45 kDa form has been observed in plasma that is probably complexed with a carrier protein, alpha-2-macroglobulin (a2m). Murine and human IL6 show 65% sequence homology at the DNA level and 42% homology at the protein level.

IL6 is a member of a family of cytokines which also includes LIF, CNTF, Oncostatin M, IL11, and CT-1. All known members of the IL6 cytokine family induce hepatic expression of acute phase proteins.

A stable and highly bioactive designer cytokine consisting of a fusion protein between IL6 and a soluble IL6 receptor, designated h-IL6, has been used for human hematopoietic progenitor cell expansion and is useful in cases in which cells do not respond to IL6 but require a stable complex consisting of IL6 and a soluble IL6 receptor.

The human IL6 gene has a length of approximately 5 kb and contains five exons. It maps to human chromosome 7p21-p14 between the markers d7s135 and d7s370. The murine gene maps to chromosome 5. The nucleotide sequences of IL6 and G-CSF genes resemble each other in a way suggesting a possible evolutionary relationship.

The IL6 receptor (e.g., polypeptides encoded by Genbank accession no. M20566, E03515) is expressed on T-cells, mitogen-activated B-cells, peripheral monocytes and some macrophage- and B-cell-derived tumor cell types. It is not expressed in resting B-cells but is in resting T-cells. In hepatocytes the IL6 receptor expression is enhanced after treatment with IL6 or IL1. In several cell types the expression of the IL6 receptor is also enhanced by glucocorticoids. The IL6 receptor gene maps to human chromosome 1q21.

The IL6 receptor is a strongly glycosylated protein of 80 kDa and a length of 449 amino acids. It has been designated CD126. It is synthesized as a precursor of 468 amino acids. The molecular structure resembles that of receptors for M-CSF, PDGF and IL1 in that the receptor contains an immunoglobulin-like sequence domain in the aminoterminal region of the extracellular receptor domain.

The intracellular domain of the IL6 receptor has a length of approximately 82 amino acids

and does not show any homology to other proteins involved in intracellular signal transduction. Two different forms of the receptor have been described that bind IL6 with different affinities (kdis $=10^{-9}$ and 10^{-11} m) and most likely arise by post-translational modification of the same receptor protein. Biological activities of IL6 have been found also at concentrations of 10^{-13} -10^{-15} m suggesting either the existence of other high-affinity receptor conformations or the existence of further receptor molecules with higher affinities.

IL6 receptor-mediated signal transduction involves protein kinase C and also adenylate cyclase.

The complex formed between IL6 and its receptor associates with a transmembrane glycoprotein, gp130 (918 amino acids; cytoplasmic domain of 277 amino acids), that is involved in signal transduction. Binding of IL6 to its receptor leads to disulfide-linked homodimerization of gp130 and the associated activation of a tyrosine kinase as the first step of signal transduction. gp130 is expressed also in cells that do not express IL6 receptors. It has been found to be a component of other receptors, including those for IL11, LIF, Oncostatin M, and CNTF, and CT-1. This explains why LIF, CNTF, and IL6 share many biological activities although the factors themselves are not related to each other. A factor resembling STAT proteins, termed lil factor, has been found to be involved in signaling pathways of IL6, and also of IL1 and bacterial lipopolysaccharides.

A soluble form of theIL6 receptor (IL6r-sup (IL6 receptor soluble urinary protein)) has been described also that also interacts with gp130. These soluble receptors probably function as physiological regulators of cytokine activities by inhibiting receptor binding or act as transport proteins.

Some cells, including hematopoietic progenitor cells and neuronal cells, are only responsive towards a combination of IL6 and soluble IL6 receptor but not to IL6 alone.

Human IL6 is biologically active in monkeys, rats, and mice. Murine IL6 is not active in human cells. The plethora of biological activities is exemplified by the many different acronyms under which IL6 has been described. IL6 is a pleiotropic cytokine influencing antigen-specific immune responses and inflammatory reactions. It is one of the major physiological mediators of the acute phase reaction. In hepatocytes IL6 in combination with glucocorticoids induces the synthesis of metallothioneins and increases intracellular zinc levels, thus preventing CCl4-induced hepatotoxicity.

IL6 is a neurotrophic factor for cholinergic neurons that promotes their survival in culture. Some neuronal cell lines can be induced to differentiate by IL6.

IL6, like IL1, stimulates the synthesis of ACTH (corticotropin) in the pituitary.

Glucocorticoids synthesized in response to ACTH inhibit the production of IL6, IL1 and TNF-alpha *in vivo*, thus establishing a sort of negative feedback loop between the immune system and neuroendocrine functions. In astrocytes IL6 induces the synthesis of nerve growth factor (NGF).

IL6 is a B-cell differentiation factor *in vivo* and *in vitro* and an activation factor for T-cells. In the presence of IL2 IL6 induces the differentiation of mature and immature T-cells into cytotoxic T-cells. IL6 also induces the proliferation of thymocytes and probably plays a role in the development of thymic T-cells.

IL6 is capable of inducing the final maturation of B-cells into immunoglobulin-secreting plasma cells if the cells have been pre-activated by IL4. In B-cells IL6 stimulates the secretion of antibodies to such a degree that serum IgG1 levels can rise 120-400-fold.

IL6 at concentrations of only 0.002 ng/ml is one of the major autocrine growth modulator for many human myelomas. The growth of these cells can be inhibited by monoclonal antibodies directed against IL6. It can be inhibited also by the introduction of antisense oligonucleotides against IL6 or by IL4. The growth-inhibitory effects of corticosteroids on myeloma cells is probably due to the steroid-induced reduction in the expression of IL6. The growth of human IL6 dependent myeloma cells can be inhibited also by IFN-gamma. IL6 may also function as an autocrine growth modulator for other tumor types, some of which have been found to secrete IL6 constitutively. IL6 has been shown to be an autocrine modulator of growth for in vitro cervical tumor cell growth. On the other hand IL6 blocks the growth of some solid tumors such as mammary carcinomas, cervical carcinomas, human lung cancer cell lines, histiocytic lymphomas, and melanomas.

IL6 and IL3 synergise in vitro in promoting the proliferation of multipotent hematopoietic progenitor cells. IL6 is also a thrombopoietin that induces the maturation of megakaryocytes in vitro and increases platelet counts *in vivo*. In murine, but not in human bone marrow cultures IL6 shows activities resembling those of GM-CSF.

Plasmacytoma cells produce IL6 and also the IL6 receptor. It has been suggested that these cells are stimulated in an autocrine fashion. A paracrine mechanism involving the presence of two different cell populations, one producing the factor and the other expressing the receptor, has been described also.

IL6 can be detected in bioassays employing IL6 responsive cell lines (e.g., 7td1; b9; cess, kpmm2, kt-3; m1, mh60-bsf-2, mo7e; mono mac 6; nfs-60; pil-6; skw6-cl4; t1165; xg-1). IL6 can be assayed also by its activity as a hybridoma growth factor. Sensitive immunoassays and colorimetric tests are also available. An elisa assay exists for detecting the receptor-associated gp130 protein.

In combination with other cytokines (for example, IL2) IL6 may be useful in the treatment of some tumor types. The transduction of murine tumor cells with a functional IL6 gene has been shown to lead to the rejection of the genetically modified cells by syngeneic hosts. Altered tumor cells expressing IL6 also increase systemic immunity. Mice vaccinated with transduced cells reject a subsequent challenge of non-transduced cells, and, in some cases, a pre-existing tumor.

Human IL10 is a homodimeric protein with subunits having a length of 160 amino acids. Human IL10 shows 73% amino acid homology with murine IL10. The human IL10 contains four exons. It is closely related to the product of the BCRF-1 gene (BamHI c fragment rightward reading frame) of Epstein-Barr virus (84 % homology at the protein level). These two proteins are more closely related to each other than human and murine IL10. BCRF-1 has therefore also been called viral IL10 (vIL10). The human IL10 gene maps to chromosome 1. The human IL10 shows 81% homology with murine IL10 at the nucleotide level.

A receptor has been identified on murine and human cells by using radiolabeled IL10 (e.g., polypeptides encoded by Genbank accession no. L12120, u00672). Mouse IL10 is capable of blocking binding of human IL10 to mouse but not human cells. The murine IL10 receptor has been cloned. This receptor is a protein of approximately 110 kDa that binds murine IL10 specifically. This receptor is structurally related to receptors for IFN.

IL10 inhibits the synthesis of a number of cytokines such as IFN-gamma, IL2 and TNF-beta in Th1 subpopulations of T-cells but not of Th2 cells. This activity is antagonized by IL4. The inhibitory effect on IFN-gamma production is indirect and appears to be the result of a suppression of IL12 synthesis by accessory cells. In the human system, IL10 is produced by, and down-regulates the function of, Th1 and Th2 cells. In macrophages stimulated by bacterial lipopolysaccharides IL10 inhibits the synthesis of IL1, IL6 and TNF-alpha by promoting, among other things, the degradation of cytokine mRNA. It also leads to an inhibition of antigen presentation. In human monocytes IFN-gamma and IL10 antagonize each other's production and function. IL10 has been shown also to be a physiologic antagonist of IL12.

IL10 also inhibits mitogen- or anti-CD3-induced proliferation of T-cells in the presence of accessory cells and reduces the production of IFN-gamma and IL2. Exogenous IL2 and IL4 inhibit the proliferation-inhibitory effect but do not influence the production of IFN-gamma. In LPS-stimulated macrophages IFN-gamma increases the synthesis of IL6 by inhibiting the production of IL10. IL10 appears to be responsible for most or all of the ability of Th2 supernatants to inhibit cytokine synthesis by Th1 cells.

IL10 inhibits secretion of Ig by T-cell-independent antigens induced by IL5 but not that induced by IL2.

Murine Ly-1 B cells are the principal source of IL10. In contrast to other B-cells, Ly-1 B-cells express greatly elevated constitutive and inducible levels of IL10. These cells also have the distinctive property of continuous self-replenishment. The continuous treatment of newborn mice with anti-IL10 antibodies leads to a depletion of the Ly-1 B-cells while maintaining a normal population of splenic B-cells. These mice also contain greatly reduced serum immunoglobulin M levels and are also impaired in their antibody responses to specific antigens. IL10 is therefore a regulator of Ly-1 B-cell development. The mechanism of Ly-1 B-cell depletion appears to involve the increased production of IFN-gamma since coadministration of neutralizing anti-IFN-gamma antibodies substantially restores the number of peritoneal-resident Ly-1 B-cells in these mice.

IL10 is also a costimulator for the growth of mature and immature thymocytes (together with IL2, IL4 and IL7) and functions as a cytotoxic T-cell differentiation factor, promoting a higher number of IL2-activated cytotoxic t-lymphocyte precursors to proliferate and differentiate into cytotoxic effector cells. IL10 sustains viability of B-cells in vitro and also stimulates B-cells and promotes their differentiation. It enhances the expression of MHC class II antigens on B-cells whereas it inhibits MHC class II expression on monocytes. In B-cells activated via their antigen receptors or via CD40 IL10 induces the secretion of IgG, IgA and IgM. This effect is synergised by IL4 while the synthesis of immunoglobulins induced by IL10 is antagonized by TGF-beta. The activation of macrophages can be prevented by IL10.

It has been shown that human IL10 is a potent and specific chemoattractant for human t-lymphocytes. The chemotactic activity is directed towards cells expressing CD8 and not towards CD4 (+)cells. IL10 also inhibits the chemotactic response of CD4 (+)cells, but not of CD8 (+)cells, towards IL8.

IL10 can be detected with a sensitive elisa assay. The murine mast cell line d36 can be used to bioassay human IL10. The intracellular factor can be detected also by flow cytometry.

The introduction of an IL10 expression vector into CHO cells has been used to analyze the consequences of local IL10 production *in vivo*. These altered cells were no longer tumorigenic in nude mice or severe combined immunodeficient scid mice and also suppressed the growth of equal numbers of co-injected normal CHO cells. While normal CHO tumors are usually substantially infiltrated by macrophages, these were virtually absent within CHO-IL10 tumor tissues, suggesting that IL10 indirectly suppresses tumor growth of certain tumors by inhibiting infiltration of macrophages which may provide tumor growth promoting activity.

Human IL12 is a heterodimeric 70 kDa glycoprotein consisting of a 40 kDa subunit (p40, 306 amino acids; 10% carbohydrate) and a 35 kDa subunit (p35, 197 amino acids; 20% carbohydrate) linked by disulfide bonds that are essential for the biological activity of IL12. p40 contains 10 cysteines and a binding site for heparin; p35 contains 7 cysteines.

The two subunits of IL12 are not related to any other known proteins. p40 shows some homology with the extracellular domain of the receptor for IL6, and p35 appears to be a homologue of IL6.

Bioactive murine and human IL12 fusion proteins combining the two IL12 subunits in a single molecule have been described. This designer cytokine retains antitumor activity *in vivo*. Flexi 12, a single chain protein retaining all of the biological characteristics of the dimeric recombinant IL12, has also been described.

The gene encoding the p40 subunit of IL12 (IL12b) maps to human chromosome 5q31-q33 in the same region that also harbors other cytokine genes. The gene encoding the p35 subunit of IL12 (IL12a) maps to human chromosome 3p12-q13.2. The expression of the two genes is regulated independently of each other.

The IL12 receptor appears to be a single protein of approximately 110 kDa (e.g., polypeptides encoded by Genbank accession no. U03187, u23922, u64198, u64199). Up to 1000-9000 high affinity IL12 receptors/cell are expressed on peripheral blood mononuclear cells activated by various T-cell mitogens or by IL2. Il12 receptors are present on activated T-cells expressing CD4 and CD8 and on activated CD56 positive natural killer cells. Resting peripheral blood mononuclear cells, tonsillar B-cells, or tonsillar B-cells activated by anti-igm/dx, anti-igm/dx +IL2, or sac +IL2 do not express the receptor. High affinity IL12 receptors are expressed constitutively on a transformed marmoset NK-like cell line, hvs.silva 40.

Binding of IL12 to its receptor can be prevented by monoclonal antibodies directed against the p40 subunit which therefore contains the binding site. The p40 subunit of IL12 shows homology with the extracellular domain of the IL6 receptor. A virus-encoded homologue of the p40 subunit is EBV-induced gene-3.

Human IL12 is not active in murine lymphocytes. Hybrid heterodimers consisting of murine p35 and human p40 subunits retain bioactivity on murine cells; however, the combination of human p35 and murine p40 is completely inactive on murine cells. Murine IL12 is active on both murine and human lymphocytes.

The p40 subunit of murine IL12 subunit p40 (IL12p40) has been shown to specifically antagonize the effects of the IL12 heterodimer in different assay systems and to function as an

endogenous specific inhibitor for the IL12 heterodimer.

Il12 stimulates the proliferation of human lymphoblasts activated by phytohemagglutinin. Il12 activates NK-cells positive for CD56, and this activity is blocked by antibodies specific for TNF-alpha. Il12 promotes specific allogenic CTL reactions. Il12 synergizes also with anti-CD3 antibodies and with allogeneic stimulation in mixed lymphocyte cultures in inducing T-cell proliferation.

In peripheral lymphocytes of the Th1 type IL12 induces the synthesis of IFN-gamma and IL2, and TNF. TNF-alpha also appears to be involved in mediating the effects of IL12 on natural killer cells since the effects of IL12 are inhibited by an antibody directed against TNF-alpha. Il12 and TNF-alpha are costimulators for IFN-gamma production with IL12 maximizing the IFN-gamma response; the production of IL12, TNF, and IFN-gamma is inhibited by IL10. In Th2 helper cells IL12 reduces the synthesis of IL4, IL5, and IL10.

Il12 synergises with suboptimal amounts of IL2 in promoting the proliferation of mononuclear cells in the peripheral blood and in promoting the generation of lak cells (lymphokine activated killer cells). Picomolar concentrations of IL12 are as effective as nanomolar concentrations of IL2 in augmenting the cytolytic activity of natural killer cells expanded *in vivo* by IL2. Il12 also acts as a co-mitogen and potentiates the proliferation of resting peripheral cells induced by IL2.

IL12 enhances myelopoiesis of primitive bone marrow progenitor cells induced by SCF (stem cell factor) and synergizes with colony stimulating factors to induce proliferation. Il12 also has synergistic effects on more committed bone marrow progenitors, synergising with IL3, IL11, or IL3 plus SCF.

Il12 is of potential clinical interest since it allows the reduction of doses of IL2 required for the generation of LAK cells (lymphokine-activated killer cells). Il12 has been shown to inhibit the growth of a variety of experimental tumors *in vivo* and to have antiangiogenic effects *in vivo*, which are, at least in part, mediated by IFN-gamma. Il12 therefore seems to be a potential candidate also for the treatment of angiogenesis-dependent malignancies.

The members of the TNF ligand superfamily (TNFalpha, TNF-beta, lymphotoxin (LT) beta, CD27 ligand, CD30 ligand, CD40 ligand, CD95 ligand, 4 LBB, OX40 ligand, TRAIL) share common biological activities, but some properties are shared by only some ligands, while others are unique. Human TNF-alpha is a non-glycosylated protein of 17 kDa and a length of 157 amino acids. Murine TNF-alpha is N-glycosylated. Homology with TNF-beta is approximately 30%. TNF-alpha forms dimers and trimers.

The 17 kDa form of the factor is produced by processing of a precursor protein of 233 amino acids. A TNF-alpha converting enzyme has been shown to mediate this conversion. A transmembrane form of 26 kDa has been described also.

TNF-alpha contains a single disulfide bond that can be destroyed without altering the biological activity of the factor. Mutations ala84 to val and val91 to ala reduce the cytotoxic activity of the factor almost completely. These sites are involved in receptor binding. The deletion of 7 N-terminal amino acids and the replacement of pro8ser9asp10 by arglysarg yields a mutated factor with an approximately 10-fold enhanced antitumor activity and increased receptor binding, as demonstrated by the l-m cell assay, while at the same time reducing the toxicity.

The gene has a length of approximately 3.6 kb and contains four exons. The primary transcript has a length of 2762 nucleotides and encodes a precursor protein of 233 amino acids. The aminoterminal 78 amino acids function as a presequence.

The human gene maps to chromosome 6p23-6q12. The gene encoding TNF-beta is approximately 1.2 kb downstream of the TNF-alpha gene. However, both genes are regulated independently. The two genes also lie close to each other on murine chromosome 17.

Approximately 500-10000 high-affinity receptors (Ka =2.5x 10^{-9} m) for TNF-alpha are expressed on all somatic cell types with the exception of erythrocytes.

Two receptors of 55 kDa (TNF-R1; new designation: CD120a) (e.g., polypeptides encoded by Genbank accession no. X55313) and 75 kDa (TNF-R2; new designation: CD120b) (e.g., as described in Goodwin RG et al., (1991) Molecular Cellular Biology 11: 3020-6) have been described. One receptor is a glycosylated protein of 455 amino acids that contains an extracellular domain of 171 and a cytoplasmic domain of 221 amino acids. Sequence homologies in the cysteine-rich domains of the extracellular portion reveal that the receptor is related to the low-affinity receptor of ngf and to human cell surface antigen CD40.

Deletion analysis in the c-terminal intracellular region of the 55 kDa receptor, TNF-R1 has revealed the existence of a so-called death domain, which is involved in signaling processes leading to programmed cell death. The death domain of TNF-R1 interacts with a variety of other signaling adaptor molecules, including TRADD, and RIP.

The two known receptors bind both TNF-alpha and TNF-beta. p55 is expressed particularly on cells susceptible to the cytotoxic action of TNF. p75 is also present on many cell types, especially those of myeloid origin (a virus-encoded homologue of the receptor subunit is EBV-induced gene-6). It is strongly expressed on stimulated T-cells and B-lymphocytes. The differential activities of TNF on various cell types, i. e. growth-promoting and growth-inhibiting activities, are probably mediated

by the differential expression and/or regulation of multiple receptors in combination with other distinct receptor-associated proteins. p55 appears to play a critical role in host defenses against microorganisms and their pathogenic factors.

A third receptor subtype is expressed in normal human liver. It binds TNF-alpha but not TNF-beta. Some viruses contain genes encoding secreted proteins with TNF binding properties that are closely homologous to the p55 and p75 TNF receptors.

Differential effects of the two receptor subtypes have been found also in TNF-mediated adhesion of leukocytes to the endothelium. It appears that engagement of the p55 receptor specifically leads to the induction of the cellular adhesion molecules ICAM-1, E-selectin, VCAM-1, and CD44, while engagement of both the p55 and the p75 receptor induces expression of alpha-2 integrin.

Truncated soluble forms of the receptor have been found also. The soluble forms, in particular the soluble extracellular domain of the p60 receptor, block the antiproliferative effects of TNF and, therefore, may modulate the harmful effects of TNF.

Receptor densities are reduced by IL1 and tumor promoters such as phorbol esters. The expression of TNF-alpha receptor density is induced by IFN-alphalpha, IFN-beta, and IFN-gamma.

Signal transducers that associate with the cytoplasmic domains of members of the TNF receptor superfamily comprise TRAF (tumor necrosis factor receptor-associated factors).

Human TNF-alpha is active on murine cells with a slightly reduced specific activity. In general, TNF-alpha and TNF-beta display similar spectra of biological activities in in vitro systems, although TNF-beta is often less potent or displays apparent partial agonist activity.

TNF-alpha shows a wide spectrum of biological activities. It causes cytolysis and cytostasis of many tumor cell lines in vitro. Sensitive cells die within hours after exposure to picomolar concentrations of the factor and this involves, at least in part, mitochondria-derived second messenger molecules serving as common mediators of TNF cytotoxic and gene-regulatory signaling pathways. The factor induces hemorrhagic necrosis of transplanted tumors. Within hours after injection TNF-alpha leads to the destruction of small blood vessels within malignant tumors. The factor also enhances phagocytosis and cytotoxicity in neutrophilic granulocytes and also modulates the expression of many other proteins, including fos, myc, IL1 and IL6.

The 26 kDa form of TNF is found predominantly on activated monocytes and T-cells. It is also biologically active and mediates cell destruction by direct cell-to-cell contacts.

The chemotactic properties of fmlp (formyl-met-leu-phe) for neutrophils are enhanced by TNF-alpha. TNF-alpha induces the synthesis of a number of chemoattractant cytokines, including

IP-10, JE, KC, in a cell-type and tissue-specific manner.

TNF-alpha is a growth factor for normal human diploid fibroblasts. It promotes the synthesis of collagenase and prostaglandin e2 in fibroblasts. It may also function as an autocrine growth modulator for human chronic lymphocytic leukemia cells *in vivo* and has been described to be an autocrine growth modulator for neuroblastoma cells. The autocrine growth-promoting activity is inhibited by IL4.

In resting macrophages TNF induces the synthesis of IL1 and prostaglandin e2. It also stimulates phagocytosis and the synthesis of superoxide dismutase in macrophages. TNF activates osteoclasts and thus induces bone resorption.

In leukocyte and lymphocyte progenitors TNF stimulates the expression of class i and ii hla and differentiation antigens, and the production of IL1, colony stimulating factors, IFN-gamma, and arachidonic acid metabolism. It also stimulates the biosynthesis of collagenases in endothelial cells and synovial cells.

Il6 suppresses the synthesis of IL1 induced by bacterial endotoxins and TNF, and the synthesis of TNF induced by endotoxins.

The neurotransmitter SP (substance p) induces the synthesis of TNF and IL1 in macrophages. Il1, like IL6, stimulates the synthesis of ACTH (corticotropin) in the pituitary. Glucocorticoids synthesized in response to ACTH in turn inhibit the synthesis of IL6, IL1 and TNF *in vivo*, thus establishing a negative feedback loop between the immune system and neuroendocrine functions.

TNF-alpha enhances the proliferation of T-cells induced by various stimuli in the absence of IL2. Some subpopulations of T-cells only respond to IL2 in the presence of TNF-alpha. In the presence of IL2 TNF-alpha promotes the proliferation and differentiation of B-cells.

The functional capacities of skin Langerhans cells are also influenced by TNF-alpha. These cells are not capable of initiating primary immune responses such as contact sensibilisation. They are converted into immunostimulatory dendritic cells by GM-CSF and also IL1. These cells therefore are a reservoir for immunologically immature lymphoid dendritic cells. The enhanced ability of maturated langerhans cells to process antigens is significantly reduced by TNF-alpha.

Although TNF-alpha is also required for normal immune responses the overexpression has severe pathological consequences. TNF-alpha is the major mediator of cachexia observed in tumor patients (hence its name, cachectin). TNF is also responsible for some of the severe effects during gram-negative sepsis.

TNF-alpha can be detected in bioassays involving cell lines that respond to it (e.g., bt-20, ct6, el4; pk15; l929; l-m; mo7e; t1165; wehi-3b). TNF-alpha can be detected also by a sensitive

sandwich enzyme immunoassay, ELISA, an immunoradiometric assay (IRMA), and by an assay designated relay (receptor-mediated label-transfer assay). Intracellular factor is detected by two color immunofluorescence flow cytometry. Higuchi et al., have described an assay based on the release of tritiated thymidine from cells undergoing apoptosis after treatment with either TNF-alpha or TNF-beta. IFN-alphalpha, IFN-beta, IFN-gamma, TGF-beta, IL4, LIF and GM-CSF have been shown not to interfere with this assay.

In contrast to chemotherapeutic drugs TNF specifically attacks malignant cells. Extensive preclinical studies have documented a direct cytostatic and cytotoxic effect of TNF-alpha against subcutaneous human xenografts and lymph node metastases in nude mice, as well as a variety of immunomodulatory effects on various immune effector cells, including neutrophils, macrophages, and T-cells. Single- and multiple-dose phase I studies have confirmed that TNF can be administered safely to patients with advanced malignancies in a dose range associated with anticancer effect without concomitant serious toxicities such as shock and cachexia. However, clinical trials on the whole have unfortunately so far failed to demonstrate significant improvements in cancer treatment, with TNF-induced systemic toxicity being a major limitation for the use of TNF as an antineoplastic agent in most cases. The combined use of TNF and cytotoxic or immune modulatory agents, particularly IFN-gamma and possibly IL2, may be of advantage in the treatment of some tumors. In some cases intratumoral application of TNF has been found to be of advantage in tumor control.

Some mutant forms of TNF-beta with selective activity on the p55 receptor have been described recently. It has been shown that activation of the p55 receptor is sufficient to trigger cytotoxic activity towards transformed cells. Some of these mutants have been described to retain their antitumor activity in nude mice carrying transplanted human tumors.

TNF can also be used to increase the aggressiveness of lymphokine-activated killer cells.

Studies with an experimental fibrosarcoma metastasis model have shown that TNF induces significant enhancement of the number of metastases in the lung. It has been suggested that low doses of endogenous TNF or administration of TNF during cytokine therapy may enhance the metastatic potential of circulating tumor cells. The transduction of murine tumor cells with a functional TNF-alpha gene has been shown to lead to the rejection of the genetically modified cells by syngeneic hosts.

The interferons are a family of cytokines that induce a virus-nonspecific antiviral state in target cells. Binding of an interferon to its receptor induces new protein synthesis which, in turn, results in the inactivation of initiation factor eIF-2. The inactivation is thought to contribute to the antiviral state induced by the interferons. Interferons also induce pathways that activate intracellular

endonucleases which degrade viral mRNA. Many interferons also possess immunomodulatory activities, such as activation of macrophages and lymphocytes. Examples of interferons include IFN-alphalpha (e.g., polypeptides encoded by Genbank Accession No. K01900, J00209, M12350, J00213, J00216, J00214, M11003, M11026, M34913, M54886, X01974, L38698, M13710, K01238, M13660, M68944, X01972, X01971, X01973, X01969), IFN-beta (e.g., polypeptides encoded by Genbank Accession No. M28622, X14029, X14455, K00020, J00218, E00171, X04430, A09363, M27327, M16656, M25460, K03196), IFN-gamma (e.g., polypeptides encoded by Genbank Accession No. A34532, X87308, E00756, K00083), IFN-omega (e.g., polypeptides encoded by Genbank Accession No. X58822, A12140), bovine trophoblast protein-1 (IFN-tau) (e.g., polypeptides encoded by Genbank Accession No. M31556, M31557, M31558), and their homologues among species. Human IFN-alpha and IFN-beta are thought to bind to a common receptor (e.g., polypeptides encoded by Genbank Accession No. X60459, M89641) which is distinct from the receptor for IFN-gamma (e.g., polypeptides encoded by Genbank Accession No. J03143, M28233).

At least 23 different variants of IFN-alpha are known. The individual proteins have molecular masses between 19-26 kDa and consist of proteins with lengths of 156-166 and 172 amino acids. All IFN-alpha subtypes possess a common conserved sequence region between amino acid positions 115-151 while the amino-terminal ends are variable. Many IFN-alpha subtypes differ in their sequences at only one or two positions. Naturally occurring variants also include proteins truncated by 10 amino acids at the carboxy-terminal end. Disulfide bonds are formed between cysteines at positions 1/98 and 29/138. The disulfide bond 29/138 is essential for biological activity while the 1/98 bond can be reduces without affecting bioactivity.

Human IFN-beta is a glycoprotein (approximately 20% sugar moiety) of 20 kDa and has a length of 166 amino acids. Glycosylation is not required for biological activity in vitro. The protein contains a disulfide bond Cys31/141) required for biological activity. At the DNA level IFN-beta displays 34% sequence homology with IFN-beta-2 and approximately 30% homology with other IFN-alpha subtypes. In contrast to IFN-gamma IFN-beta is stable at pH2.

Human IFN-gamma is a dimeric protein with subunits of 146 amino acids. The protein is glycosylated at two sites. The pI is 8.3-8.5. IFN-gamma is synthesized as a precursor protein of 166 amino acids including a secretory signal sequence of 23 amino acids. Two molecular forms of the biologically active protein of 20 and 25 kDa have been described. Both of them are glycosylated at

position 25. The 25 kDa form is also glycosylated at position 97. The observed differences of natural IFN-gamma with respect to molecular mass and charge are due to variable glycosylation patterns. 40-60 kDa forms observed under non-denaturing conditions are dimers and tetramers of IFN-gamma.

Members of the CSF family of cytokines allow the growth and differentiation of bone marrow cells immobilized on soft agar or methylcellulose. While hematopoietic progenitor cells can be maintained only for short periods of time in the absence of such factors, their presence allows the development of colonies containing erythroid cells, neutrophils, eosinophils, macrophages, and/or megakaryocytes, depending on the particular factor. The biochemical analysis of various activities stimulating colony formation supporting the growth and development of these cell types revealed that there existed many different and distinct factors of this sort.

Many of these factors are either N - or O-glycosylated. Glycosylation has been shown to enhance the solubility, stability and resistance to proteolytic enzymes. It does not appear to be required for the full spectrum of biological activities of these factors. The genes encoding many of the human colony stimulating factors have been cloned and mapped. Some of the genes are in close vicinity but they do not show great homology among each other with the exception of some conserved regions.

Colony stimulating factors are produced by many different cell types, including, for example, B-lymphocytes, epithelial cells, fibroblasts, endothelial cells, macrophages, Stromal cell line, T-lymphocytes. They are synthesized as precursor molecules containing a classical hydrophobic secretory signal sequence of approximately 25-32 amino acids. The secreted factors have an extremely high specific biological activity are active at very low concentrations (1-100 pM). These factors are absolutely required for the proliferation of hematopoietic progenitor cells. The concentrations required for mere maintenance of viability are usually orders of magnitude lower than those required to induce cell proliferation or to elicit specific functional activities of the cells.

The names of the individual factors usually indicate the cell types that respond to these factors. The classical colony stimulating factors include M-CSF (e.g., polypeptides encoded by Genbank Accession No. E03235, M64592, U22386, X05010) (macrophage-specific), G-CSF (granulocyte-specific), GM-CSF (macrophage/granulocyte-specific), IL3 (multifunctional) and MEG-CSF (e.g., polypeptides encoded by Genbank Accession No.D86370, U70136) (megakaryocyte-specific). G-CSF and M-CSF are lineage-specific while GM-CSF and IL3 are multifunctional hematopoietic growth factors acting on earlier stages of differentiation of

hematopoietic progenitor cells.

Human GM-CSF is a monomeric protein of 127 amino acids with two glycosylation sites. The protein is synthesized as a precursor of 144 amino acids, which included a hydrophobic secretory signal sequence at the aminoterminal end. The sugar moiety is not required for the full spectrum of biological activities. Non-glycosylated and glycosylated GM-CSF show the same activities in vitro. Fully glycosylated GM-CSF is biologically more active *in vivo* than the non-glycosylated protein. The different molecular weight forms of GM-CSF (14 kDa, 35 kDa) described in the literature are the result of varying degrees of glycosylation. GM-CSF contains four cysteine residues (positions 54/96 and 88/121).

A comparison of the protein sequence of GM-CSF with those of the other colony stimulating factors reveals that they are not strongly homologous to each other. Human and murine GM-CSF display 60% homology at the protein level and 70% at the nucleotide level. The two factors do not, however, cross-react immunologically.

GM-CSF can be associated with the extracellular matrix of cells as a complex with heparan sulfate proteoglycans. This allows storage of the factor in a biologically inactive form. The exact mechanism by which the factor is eventually released from these depots is not known.

The human gene has a length of approximately 2. 5 kb and contains four exons. The distance between the GM-CSF gene and the IL3 gene is approximately 9 kb.

The human GM-CSF gene maps to chromosome 5q22-31 in the vicinity of other genes encoding hematopoietic growth factors (M-CSF, IL3, IL4, IL5) and the gene encoding the M-CSF receptor. The 5' region of the GM-CSF gene contains several sequence elements known as CLE (conserved lymphokine element). They function as binding sites for transcription factors, modulating the expression of the GM-CSF gene.

GM-CSF receptors are expressed at densities of several 100 to several 1000 copies/cell on the cell surface of myeloid cells. The receptor is expressed also on non-hematopoietic cells such as endothelial cells and small cell lung carcinoma cells.

In receptor-positive cell lineages the receptor density decreases with increasing degrees of maturation.

The receptor shows significant homologies with other receptors for hematopoietic growth factors, including IL2-beta, IL3, IL6, IL7, Epo and the prolactin receptors. One cloned subunit of the GM-CSF receptor (GM-R alpha, 45 kDa) binds GM-CSF with low affinity (e.g., polypeptides encoded by Genbank Accession No. SEG_HUMGRAS). The second subunit (GM-R beta, 120 kDa)

does not bind GM-CSF. GM-R alpha is a protein of 400 amino acids that contains only a short cytoplasmic domain of 54 amino acids. The high affinity GM-CSF receptor is formed by the aggregation of the two receptor subunits. The GM-R beta subunit of the receptor (e.g., polypeptides encoded by Genbank Accession No. SEG_MUSAIC2B, M59941) is also a constituent of other cytokine receptor systems. It is a component of the high affinity receptors for IL3 and IL5, both of which also contain a cytokine-specific subunit (AIC2A).

Human GM-CSF is not active on murine cells and vice versa. GM-CSF was isolated initially as a factor stimulating the growth of macrophage/granulocyte-containing colonies in soft agar cultures (colony formation assay). GM-CSF is indispensable for the growth and development of granulocyte and macrophage progenitor cells. It stimulates myeloblasts and monoblasts and triggers irreversible differentiation of these cells. GM-CSF synergises with Epo in the proliferation of erythroid and megakaryocytic progenitor cells. In combination with another colony stimulating factor, M-CSF, one observes the phenomenon of synergistic suppression, i.e., the combination of these two factors leads to a partial suppression of the generation of macrophage-containing cell colonies.

For some types of blast cells from patients with acute myeloid leukemia GM-CSF acts as an autocrine mediator of growth. GM-CSF is a strong chemoattractant for neutrophils. It enhances microbicidal activity, oxidative metabolism, and phagocytotic activity of neutrophils and macrophages. It also improves the cytotoxicity of these cells.

GM-CSF displays a less pronounced specificity than, for example, G-CSF. It stimulates the proliferation and differentiation of neutrophilic, eosinophilic, and monocytic lineages. It also functionally activates the corresponding mature forms, enhancing, for example, the expression of certain cell surface adhesion proteins (CD-11A, CD-11C). The overexpression of these proteins could be one explanation for the observed local accumulation of granulocytes at sites of inflammation. In addition, GM-CSF also enhances expression of receptors for fMLP (Formyl-Met-Leu-Phe) which is a stimulator of neutrophil activity.

At pico- to nanomolar concentrations GM-CSF is chemotactic for eosinophils and also influences the chemotactic behavior of these cells in response to other chemotactic factors.

In granulocytes GM-CSF stimulates the release of arachidonic acid metabolites and the increased generation of reactive oxygen species. The activation of the Na+/H+ antiport system leads to a rapid alkalization of the cytosol. Phagocytotic activities of neutrophil granulocytes and the cytotoxicity of eosinophils is also enhanced considerably by GM-CSF. Since GM-CSF is produced by cells (T-lymphocytes, tissue macrophages, endothelial cells, mast cells) present at sites of

inflammatory responses it can be assumed that it is an important mediator for inflammatory reactions.

The functional state of Langerhans cells of the skin is also influenced by GM-CSF. These cells are not capable of initiating primary immune responses, for example, contact sensibilization. They are converted to highly potent immunostimulatory dendritic cells by GM-CSF (and also IL1). Langerhans cells therefore form an in situ reservoir for immunologically immature lymphoid dendritic cells. The maturation of these cells which is seen as an increased ability to process antigens, can be down-regulated by TNF-alpha.

At nanomolar concentrations GM-CSF induces the expression of complement C3a receptors on basophils. Cells which normally do not respond to C3a and which have been activated by GM-CSF degranulate in response to the C3a stimulus. This is accompanied by the release of histamine and leukotriene C4. This process may be of significance in hypersensitivity reactions associated with inflammatory responses (T-lymphocytes, tissue macrophages, endothelial cells, mast cells). GM-CSF has been shown also to be a potent inducer of trophoblast interferon (TP-1).

GM-CSF synergises with some other cytokines, including IL1, IL3 and G-CSF. GM-CSF and G-CSF must act in concert to allow the development of neutrophil-containing colonies in vitro.

IL3 by itself only negligibly expands the number of circulating blood cells; a subsequent dose of GM-CSF, however, significantly increases cell numbers, probably because IL3 first leads to an expansion of those cells capable of responding to GM-CSF.

The observations that most IL3-dependent cell lines can also grow in the presence of GM-CSF and IL4 and that several synergistic effects are observed between GM-CSF and IL4 suggest that these three factors perform similar functions in controlling the growth of cells. There are some indications that the mechanism of signal transduction contains at least some common factors.

Experiments with tyrosine-specific protein kinases encoded by an oncogene have shown that the expression of this kinase activity in factor-dependent cells abolishes their dependence on GM-CSF, IL3 and IL4. The exact mechanism by which these factors regulate the proliferation and differentiation of cells is still unknown.

The consequences of a deregulated expression of GM-CSF have been studied in transgenic mice harboring a constitutively expressed GM-CSF gene. The overexpression of the transgene encoding GM-CSF leads to pathological alterations in the retina and causes blindness and also causes muscle deterioration. These mice are characterized by a very pronounced increase in activated macrophages. In addition, the overexpression of GM-CSF leads to the activation of mature

macrophages secreting large amounts of IL1 and TNF, suggesting that these cytokines may be responsible for some aspects of the transgenic mouse disease.

Histopathological examination demonstrates a pronounced increase in the progenitor cell population of the monocytic lineage. GM-CSF-transgenic animals usually die within months from the massive tissue damage resulting from the overexpression of these factors. Similar results have been obtained with mice possessing a bone marrow manipulated to overexpress GM-CSF by transformation with suitable retrovirus vectors. These findings do not seem to be of clinical significance, though. The long-term treatment of primates and mice with GM-CSF has shown that life-threatening complications do not occur.

The biological consequences of GM-CSF gene disruption have been studied in mice generated from ES cells carrying a targeted deletion of the gene. Mice homozygous for a targeted disruption of the GM-CSF gene are characterized by an unimpaired steady-state hematopoiesis, demonstrating that GM-CSF is not essential for maintaining normal levels of the major types of mature hematopoietic cells and their precursors in blood, marrow, and spleen.

Most GM-CSF-deficient mice are superficially healthy and fertile but develop abnormal lungs. GM-CSF-deficient mice develop a progressive accumulation of surfactant lipids and proteins in the alveolar space, the defining characteristics of the idiopathic human disorder pulmonary alveolar proteinosis. Extensive lymphoid hyperplasia associated with lung airways and blood vessels is found also. These results demonstrate an unexpected, critical role for GM-CSF in pulmonary homeostasis.

Transgenic mice homozygous for null mutations of the gene encoding the common beta subunit (beta C) of the GM-CSF, IL3, and IL5 receptor complexes exhibit normal development and survive to young adult life. They develop pulmonary peribronchovascular lymphoid infiltrates and areas resembling alveolar proteinosis. Eosinophil numbers in peripheral blood and bone marrow of homozygous deletion mutants are reduced, while other hematological parameters are normal. Bone marrow cells from homozygous deletion mutants do not show high-affinity binding of GM-CSF, while cells from heterozygous animals show an intermediate number of high-affinity receptors. In clonal cultures of bone marrow cells derived from homozygous deletion mutants, even high concentrations of GM-CSF and IL5 do not stimulate colony formation in the colony formation assay. Differences in the systemic clearance and distribution of GM-CSF between mutant and wild-type littermates are not observed.

Nishinakamura et al., have crossed beta-c mutant mice with mice deficient for IL3. The double-mutant mice lacking all IL3, GM-CSF, and IL5 functions are apparently normally fertile. The

animals show the same reduced numbers of eosinophils and a lack of eosinophilic response to parasites as beta-c mutant mice. The immune response of the double mutant mice to Listeria monocytogenes is normal. Hematopoietic recovery after treatment with fluorouracil is also normal. These findings suggest the existence of alternative mechanism to produce blood cells that do not depend on the presence of IL3, GM-CSF, and IL5.

GM-CSF can be assayed in a colony formation assay by the development of colonies containing macrophages, neutrophils, eosinophils, and megakaryocytes. GM-CSF is also detected in specific bioassays with cells lines that depend in their growth on the presence of GM-CSF or that respond to this factor (e.g., AML-193; B6SUt-A; BAC1.2F5; BCL1; Da; FDCP1; GF-D8; GM/SO; IC-2; MO7E; NFS-60; PT-18; TALL-103; TF-1; UT-7).

GM-CSF can be employed for the physiological reconstitution of hematopoiesis in all diseases characterized either by an aberrant maturation of blood cells or by a reduced production of leukocytes. The main and most important clinical application of GM-CSF is probably the treatment of life-threatening neutropenia following chemo- and/or radiotherapy, which is markedly reduced under GM-CSF treatment. GM-CSF can be used also to correct chemotherapy-induced cytopenias and to counteract cytopenia-related predisposition to infections and hemorrhages.

In order to avoid potential complications following the administration of GM-CSF careful clinical monitoring is required in certain patient groups, for example those with myelodysplastic syndrome, acute myeloid leukemia, inflammatory disease, autoimmune thrombocytopenia or malfunctional immunological responsiveness.

Several studies have demonstrated that the use of GM-CSF enhances tolerance to cytotoxic drug treatment and can be used to prevent dose reductions necessitated by the side effects of cytotoxic drug treatment. GM-CSF treatment frequently permits to increase the doses of cytotoxic drugs per course. These studies have also revealed a significantly reduced morbidity under GM-CSF treatment.

The transduction of murine tumor cells with a functional GM-CSF gene has been shown to lead to the rejection of the genetically modified cells by syngeneic hosts. Moreover, vaccination with GM-CSF transduced tumor cells prevents growth of a subsequent inoculum of wild-type syngeneic tumor cells.

The chemokine family of cytokines consists of relatively small, structurally similar polypeptides that induce chemotaxis in leukocytes. Chemokines have molecular masses of 8-10 kDa and show approximately 20-50% sequence homology among each other at the protein level. The proteins also share common gene structures and tertiary structures. All chemokines possess a number

of conserved cysteine residues involved in intramolecular disulfide bond formation.

According to the chromosomal locations of individual genes two different subfamilies of chemokines are distinguished. Members of the alpha-chemokines are referred to also as the 4q chemokine family because the genes encoding members of this family map to human chromosome 4q12-21. The first two cysteine residues of members of this family are separated by a single amino acids and these proteins, therefore, are called also C-X-C chemokines. This subfamily includes 9E3 (e.g., Genbank protein P08317), AMCF (e.g., polypeptides encoded by Genbank Accession No. M99367, M99368), beta-thromboglobulin (e.g., as disclosed in Begg GS et al., (1978), Biochemistry 17: 1739-44), CINC family members (e.g., polypeptides encoded by Genbank Accession No. D21095), ENA-78 (e.g., polypeptides encoded by Genbank Accession No. X78686), eotaxin (e.g., polypeptides encoded by Genbank Accession No. U46572, U40672), GCP-2 (e.g., polypeptides encoded by Genbank Accession No. Y08770, U83303), IL8, IP-10 (e.g., polypeptides encoded by Genbank Accession No. L07417, X02530), KC (e.g., polypeptides encoded by Genbank Accession No. J04596), LIX (e.g., polypeptides encoded by Genbank Accession No. U27267), mig (e.g., polypeptides encoded by Genbank Accession No. M34815, Z24725), MGSA (e.g., polypeptides encoded by Genbank Accession No. X12510), mob-1 (e.g., polypeptides encoded by Genbank Accession No. U17035), NAP-2 (as described in Clark-Lewis I et al., (1991) Biochemistry 30: 3128-35, Cohen AB et al., (1992) American Journal of Physiology 263: L249-56), NAP-3 (as described in: Schröder JM et al., (1991) Journal of Experimental Medicine 171: 1091-100), NAP-4 (as described in Schröder JM et al., (1990) Biochemical and Biophysical Research Communications 172: 898-904), PBSF (SDF) (e.g., polypeptides encoded by Genbank Accession No. D21072, U16752, D50645), and PF4 (e.g., polypeptides encoded by Genbank Accession No. M25897).

IL8, MGSA, mouse KC, MIP-2 (e.g., polypeptides encoded by Genbank Accession No. X65647 and as descibed in Blum S et al., Three human homologues of a murine gene encoding an inhibitor of stem cell proliferation. DNA Cell Biol. 9: 589-602 (1990); Clements JM et al., Biological and structural properties of MIP-1 alpha expressed in yeast. Cytokine 4: 76-82 (1992); Devatelis G et al., Cloning and characterization of a cDNA for murine macrophage inflammatory protein (MIP), a novel monokine with inflammatory and chemokinetic properties. Journal of Experimental Medicine 167: 1939-44 (1988) (erratum in JEM 170: 2189 (1989)); Farber JM A macrophage mRNA selectively induced by gamma-interferon encodes a member of the platelet factor 4 family of cytokines. Proceedings of the National Academy of Science (USA) 87: 5238-42 (1990); Haskill S et al., Identification of three related human GRO genes encoding cytokine functions. Proceedings of the National Academy of Science (USA) 87: 7732-6 (1990); Poltorak AN

et al., (1995) Journal of Inflammation 45(3): 207-19; Rossi DL et al., (1997) Journal of Immunology 158(3): 1033-1036; Sherry B et al., (1988) Journal of Experimental Medicine 168: 2251-9; Tekamp-Olson P et al., (1990) Journal of Experimental Medicine 172: 911-9; Wolpe SD et al., (1989) Proceedings of the National Academy of Science (USA) 86: 612-16; Wolpe SD et al., (1989) FASEB Journal 3: 2565-73), NAP-2, ENA-78, and GCP-2 comprise a subgroup of the human C-X-C-chemokines defined by the conserved ELR sequence motif (glutamic acid-leucine-arginine) immediately preceding the first cysteine residue near the amino-terminal end. Chemokines with an ELR sequence motif have been found to chemoattract and activate primarily neutrophils. Chemokines without the ELR sequence motif appear to chemoattract and activate monocytes, dendritic cells, T-cells, NK-cells, B-lymphocytes, basophils, and eosinophils.

Members of the beta-chemokines or 17q chemokine family map to human chromosome 17q11-32 (murine chromosome11). The first two cysteine residues are adjacent and, therefore, these proteins are called also C-C chemokines. This subfamily includes ACT-2 (e.g., polypeptides encoded by Genbank Accession No. J04130), C10 (e.g., as described in Berger MS et al., (1993) DNA Cell Biol. 12: 839-47; Berger MS et al., (1996) 8: 439-447), CCF18 (e.g., as described in Hara T et al., (1995) Journal of Immunology 155: 5352-8), DC-CK1 (e.g., as described in Adema GJ et al., (1997) Nature 387: 713-717), ELC (e.g., polypeptides encoded by Genbank Accession No. AB000887, AF059208), Eotaxin-2 (e.g., as described in Forssmann U et al., (1997) Journal of Experimental Medicine 185: 2171-2176), Exodus (e.g., polypeptides encoded by Genbank Accession No. U64197, U88320, U88321, U88322), FIC (e.g., polypeptides encoded by Genbank Accession No. L04694), GDCF and GDCF-2 (e.g., as descrbed in Kuratsu J et al., (1989) Journal of the National Cancer Institute 81: 347-51; Yoshimura T et al., (1989) Journal of Experimental Medicine 169: 1449-59; Yoshimura T et al., (1989) Journal of Immunology 142: 1956-62), HC-21 (e.g., as described in Chang HC & Reinherz EL (1989) European Journal of Immunology 19:1045-1051), HCC-1 (e.g., polypeptides encoded by Genbank Accession No. Z49270), I-309 (e.g., polypeptides encoded by Genbank Accession No. M57502), JE (e.g., polypeptides encoded by Genbank Accession No. AF058786, M28226), LAG-1 (lymphocyte activation gene-1) (e.g., polypeptides encoded by Genbank Accession No. X53683), LARC D86955), LD78 E03130, E03131, MARC (e.g., as described in Thirion S et al., (1994) Biochemical and Biophysical Research Communications 201: 493-499); MCAF M24545 and as described in Apella E et al., (1990) Progress in Clinical and Biological Research 349: 405-17), MCP-1 (e.g., polypeptides encoded by Genbank Accession No. X14768), MCP-2 (e.g., polypeptides encoded by Genbank Accession No. Y16645), MCP-3 (e.g., polypeptides encoded by Genbank Accession No. X72308, S71251), MCP-4 (e.g.,

polypeptides encoded by Genbank Accession No. X98306), MCP-5 (e.g., polypeptides encoded by Genbank Accession No. U50712), MIP (macrophage inflammatory protein) (e.g., polypeptides encoded by Genbank Accession No. U77180, U77035, U49513, M35590), MRP-2 (e.g., as described in Youn BS et al., (1995) Journal of Immunology 155: 2661-7), RANTES SDF (e.g., polypeptides encoded by Genbank Accession No. M21121, M77747), TARC (e.g., Genbank protein Accession No. Q92583).

In addition there are several other factors that are related to chemokines but that either have not been assigned yet to one of the two chemokine groups or that do not possess the classical features of either of the two chemokine groups (for example, ATAC (e.g., polypeptides encoded by Genbank Accession No. X86474), Ltn (e.g., polypeptides encoded by Genbank Accession No. U15607, U23772), SCM-1 (e.g., polypeptides encoded by Genbank Accession No. D63789, D63790, D43769). These have been referred to as C-type chemokines or gamma-chemokines.

Yet another group of chemokines has been identified that comprises neurotactin (e.g., polypeptides encoded by Genbank Accession No. AF010586, which is characterized by a CX(3)C cysteine signature motif. The existence of clearly defined subgroups of chemokines on the basis of structural and functional properties illustrates the importance of chemoattractant diversity in the regulation of leukocyte movement through the body.

The biological activities of chemokines are mediated by specific receptors and also by receptors with overlapping ligand specificities that bind several of these proteins which always belong either to the C-C-chemokines or the group of C-X-C-chemokines. Chemokine receptors belong to the large group of G-protein-coupled seven transmembrane domain receptors which contain seven hydrophobic alpha-helical segments that transverse the membrane. These receptors form a structurally related group within the superfamily of G-protein-coupled receptors which mediate signaling via heterotrimeric G-proteins.

The receptors that bind C-X-C chemokines are designated CXCR followed by a number (e.g., CXCR-1 (e.g., polypeptides encoded by Genbank Accession No. L19591),CXCR-2 (e.g., polypeptides encoded by Genbank Accession No. M94582), CXCR-3 (e.g., polypeptides encoded by Genbank Accession No. X95876),CXCR-4 (e.g., polypeptides encoded by Genbank Accession No. D87747, AF025375) while those binding C-C chemokines are designated CCR followed by a number (e.g., CCR-1 (e.g., polypeptides encoded by Genbank Accession No.L09230, U29678), CCR-2 (e.g., polypeptides encoded by Genbank Accession No. U29677, U95626), CCR-3 (e.g., polypeptides encoded by Genbank Accession No. U51241), CCR-4 (e.g., polypeptides encoded by Genbank Accession No. X90862, X85740), CCR-5 (e.g., polypeptides encoded by Genbank

Accession No. U54994, U83327), CCR-6 (e.g., polypeptides encoded by Genbank Accession No. U95626), CCR-7 (e.g., polypeptides encoded by Genbank Accession No. L31581), CCR-8 (e.g., polypeptides encoded by Genbank Accession No. Z98206, U45983). Viral chemokine receptor homologues include ECRF-3, EBI-1 (EBV-induced gene-1), and US28.

It is now assumed that the combinatorial effects of multiple chemokines and other mediators are responsible for the cellular composition at inflammatory sites. In addition, many chemokines also directly activate cells. Some of them activate granulocytes and/or monocytes and cause respiratory bursts, degranulation, and the release of lysosomal enzymes. Others prime immune cells to respond to sub-optimal amounts of other inflammatory mediators. Yet others have been shown to be potent histamine releasing factors for basophils. It has been proposed that erythrocytes through their promiscuous chemokine receptor play an important role in regulating the chemokine network. Chemokines bound to the erythrocyte receptor are known to be inaccessible to their normal target cells. This appears to provide a sink for superfluous chemokines and may serve to limit the systemic effects of these mediators without disrupting localized processes taking place at the site of inflammation.

Certain C-C chemokines exhibit biological activities other than mere chemotaxis. Some chemokines have been shown to be capable of inducing the proliferation and activation of killer cells known as CHAK (C-C-chemokine-activated killer), which are similar to cells activated by IL2.

Antitumor cytokines are especially useful in the methods and compositions of the invention. According to the invention, an "antitumor cytokine" is a cytokine that can limit the growth or metastasis of tumor cells in vitro or *in vivo*, or can prolong the survival of a tumor-bearing animal, when either admixed with the cells or administered to the animal. The cytokine can be formulated as a solution in a biologically compatible buffer, e.g., PBS, and admixed with tumor cells in vitro. The concentration of cytokine may be from about the picomolar range to about the micromolar range. An antitumor cytokine will, for example, reduce the growth rate of the cells, e.g., by at least 10% compared to buffer alone, or inhibit metastatic properties of the cells, as may be evidenced by, e.g., increased cell adhesiveness or decreased ability to invade an extracellular matrix substrate, such as an artificial basement membrane. Alternatively, an antitumor cytokine may inhibit the growth or metastasis of a tumor *in vivo*, or may prolong the survival of a tumor-bearing animal. To evaluate the *in vivo* antitumor effects of a cytokine, the cytokine may be formulated in a pharmaceutically acceptable carrier and administered, e.g., by intravenous, intratumoral, or intraperitoneal injection. The cytokine may also be administered in association with cells, such as tumor cells that express or

are coated with the cytokine.

Assays for Bioactivity

According to the invention, it is preferred that a cytokine be "bioactive", "highly bioactive", "extremely bioactive", "natively bioactive", or "suprabioactive". Different levels of bioactivity relate to the ability to induce a change in a leukocyte (other than mere occupancy of the leukocyte's receptors for the cytokine). According to the invention, all naturally occurring cytokines are natively bioactive. Many types of assay can demonstrate the bioactivity of a non-naturally occurring cytokine. For example, a cytokine may be shown to induce survival and/or proliferation of a particular cell type. As another example, a cytokine may change the concentration of an intracellular second messenger, such as cAMP, arachidonic acid, calcium ions, or inositol triphosphate. The following are examples of assays for bioactivity:

Assay 1

Each well of one or more 60-well Lux microtiter trays is loaded with 200 FDC-P1 cells in 10 ul Dulbecco's modified Eagle's medium with a final concentration of 10% newborn calf serum. Cytokine in a concentration in at most the micromolar range is added to each well in a volume of 5 ul. The tray is incubated for 48h at 37°C in 10% CO_2. Viable cell counts are performed. The average number of viable cells/well is counted. This assay is useful, for example, for identifying bioactivity mediated through a murine GM-CSF receptor.

Assay 2

Cytokine sample and a recombinant standard identical to a naturally occurring cytokine are each diluted serially in complete RPMI-10 in 96-well flat-bottom microtiter plates. Each dilution is plated in triplicate. CT.4S cells in active log-phase growth are collected, washed at least twice in complete RPMI-10, and resuspended in complete RPMI-10 at 1×10^5 cells/ml. 50 ul of the cell suspension is added to each well of the plate, which is then incubated for 24h at 37°C in 5% CO_2. Tritiated thymidine is added to each well and the plate is incubated for an additional 24h. The cells are then harvested and tritium incorporation is measured by liquid scintillation counting. This assay is useful, for example, for identifying bioactivity mediated through an IL-4 receptor.

Assay 3 (Colony Formation Assay)

Agar (4% w/v) is melted in sterile water by boiling 3 min. The agar is then cooled to 42°C

and added to 42°C RPMI-15 to a final concentration of 0.4%. The solution is maintained at 42°C. Femurs are removed from young mice using sterile technique. Marrow is collected by flushing the opened ends of the bones with sterile Hank's Balanced Salt Solution (HBSS) using a syringe equipped with a 23G needle. Marrow is placed in a 15 ml tissue culture tube and vortexed into a cell suspension. Bone fragments are allowed to settle for 5 min, and the supernatant suspension is removed. The suspension is adjusted to 7.5 x 10^6 nucleated cells/ml and diluted 1:100 by adding the 42° C RPMI with 0.4% agar. 2-fold serial dilutions of cytokine are added to 35 mm tissue culture dishes in a volume < = 0.2 ml. Control dishes have no cytokine added. 1 ml warm cell suspension is added to each dish and the agar is allowed to set at room temperature. The cultures are incubated for 5-7 days at 37° C in 5% CO_2. Colony formation is then evaluated by microscopy. The average number of colonies of a given type (or aggregate number of colonies of given different types) on the cytokine plates and the average number on the control plates is counted. This assay is useful, for example, for identifying bioactivity mediated through CSF receptors.

Assay 4

Cytokine is diluted serially in RPMI 1640/25 mM HEPES/1% BSA. 25 ul of each dilution is plated in triplicate in a multiwell chemotaxis chamber bottom. Wells containing medium alone serve as negative controls and wells containing chemotaxis-inducing naturally occurring cytokine serve as positive controls. A polycarbonate membrane is placed over the chamber bottom and the chamber is assembled. 50 ul of peripheral blood mononuclear cells at 1.5 x 10^6 cells/ml in the RPMI/HEPES/BSA is added to each of the upper wells of the chamber. The chamber is incubated for 90 min at 37°C in 5% CO_2. The membrane is removed, washed, and stained. Migrated cells in 3-5 random fields of each well are counted by microscopy.

Assay 5

Naturally-occurring cytokine reference standard is diluted to 2 ng/ml in a 17 x 100 mm tube using supplemented medium. 3 further 5-fold serial dilutions are also prepared. Serial dilutions of cytokine are prepared in 17 x 100 mm tubes from 2 ng/ml to 20 pg/ml. 50 ul of PHA-activated human lymphoblasts 4 x 10^5 cells/ml in supplemental medium is added to each well of a 96-well flat-bottom microtiter plate. 50 ul of each dilution of reference standard or cytokine is added to triplicate wells. Negative control wells receive 50 ul of supplemented media alone. The plate is incubated for 48h at 37°C in 5% CO_2 and the cells are labeled with tritiated thymidine

Incorporation is measured by liquid scintillation counting. This assay is useful, for example, for identifying bioactivity mediated through an IL-12 receptor.

Assay 6

In another assay for bioactivity, an immunocompetent animal is vaccinated with on the order of $10^4 10^8$ irradiated cytokine-transduced or cytokine-coated tumor cells, and challenged with on the order of $10^4 10^8$ live wildtype tumor cells (in any temporal sequence). Readouts of the assay are survival, tumor onset, or number of metastases.

Further examples of cytokine assays can be found, e.g., in: Callard RE et al., Assay for human B cell growth and differentiation factors. in: Clemens MJ et al., (eds) Lymphokines and Interferons. A practical Approach, pp. 345-64, IRL Press, Oxford 1987; Coligan JE et al., Current protocols in immunology. Grene and Wiley-Interscience, New York 1991); Dotsika EN Assays for mediators affecting cellular immune functions. Current Opinion in Immunology 2: 932-5 (1989); Feldmann M et al., Cytokine assays: role in evaluation of the pathogenesis of autoimmunity. Immunological Reviews 119: 105-123 (1991); Guiguet M et al., Misinterpretation of the biological activity of cytokine-containing preparations attributable to unrecognized interacting components. Analytical Biochemistry 247(2): 441-442 (1997); Hamblin AS & O'Garra A Assays for interleukins and other related factors. In: Lymphocytes, a practical approach, Klaus GGB (edt), pp. 209-28, IRL Press, Oxford, (1987); Laska EM & Meisner MJ Statistical methods and applications of bioassay. Annu. Rev. Pharmacol. Toxicol. 27: 385-97 (1987); Mosman TR & Fong TAT Specific assays for cytokine production by T cells Journal of Immunological Methods 116: 151-8 (1989); Newton RC & Uhl J Assays relevant to the detection and quantitation of cytokines and their inhibitors. Modern Methods in Pharmacol. 5: 83-99 (1989); Thorpe R et al., Detection and measurement of cytokines. Blood Rev. 6: 133-48 (1992); van Zoelen EJ The use of biological assays for detection of polypeptide growth factors. Progress in Growth Factor Research 2: 131-52 (1990); Winstanley FP Cytokine bioassay. In: Gallagher G et al., (eds) Tumor Immunobiology, A practical Approach. Oxford University Press, pp. 179-303 (1993); Wadha M et al., Quantitative biological assays for individual cytokines. In: Balkwill FR (edt) Cytokines, A practical approach. Oxford University press, pp. 309-330 (1991).

According to the invention, if a non-naturally occurring cytokine gives a readout in a bioactivity assay that is at least 10% but not more than 29% (to the nearest 1%) of the readout yielded by an equimolar amount of a naturally occurring cytokine (the latter giving a positive result in the assay), then the non-naturally occurring cytokine is "bioactive". According to the invention,

if a non-naturally occurring cytokine gives a readout in a bioactivity assay that is at least 30% but not more than 49% (to the nearest 1%) of the readout yielded by an equimolar amount of a naturally occurring cytokine (the latter giving a positive result in the assay), then the non-naturally occurring cytokine is "highly bioactive". According to the invention, if a non-naturally occurring cytokine gives a readout in a bioactivity assay that is at least 50% but not more than 69% (to the nearest 1%) of the readout yielded by an equimolar amount of a naturally occurring cytokine (the latter giving a positive result in the assay), then the non-naturally occurring cytokine is "extremely bioactive". According to the invention, if a non-naturally occurring cytokine gives a readout in a bioactivity assay that is at least 70% but not more than 100% (to the nearest 1%) of the readout yielded by an equimolar amount of a naturally occurring cytokine (the latter giving a positive result in the assay), then the non-naturally occurring cytokine is "natively bioactive". According to the invention, if a non-naturally occurring cytokine gives a readout in a bioactivity assay that is greater than 100% of the readout yielded by an equimolar amount of a naturally occurring cytokine (the latter giving a positive result in the assay), then the non-naturally occurring cytokine is "suprabioactive".

Ligands for CD40 Useful According to the Invention

Nucleotide sequences encoding the CD40 proteins of various species are provided by, e.g., Genbank Accession Nos. Y10507, M83312, and U57745. Human CD40 is a transmembrane glycoprotein with a length of 277 amino acids (48 kDa). CD40 is a phosphoprotein and can be expressed as a homodimer. A soluble form of CD40 (28 kDa) has also been described. CD40 protein is expressed on all B-lymphocytes during various stages of development, activated T-cells and monocytes, follicular dendritic cells, thymic epithelial cells, and various carcinoma cell lines. It is expressed on most mature B-cell malignancies and on some early B-cell acute lymphocytic leukemias. CD40 has been demonstrated on the majority of myeloma cell lines and myeloma cells from patients with plasma cell dyscrasia.

Induction of CD40 mRNA and enhancement of cell surface protein expression in primary human monocytes is observed after treatment with GM-CSF, IL3, or IFN-gamma. The human CD40 gene maps to chromosome 20.

CD40 has been proposed to play a role in the development of memory cells. It also plays a role in cell activation, functioning as a competence factor and progression factor. Crosslinking of the CD40 antigen (in combination with cytokines such as IL4 and IL5) leads to B-cell proliferation and induces immunoglobulin class switching from IgM to the synthesis of IgG, IgA, and IgE in the absence of activated T-cells. CD40 is one of the obligatory signals required for commitment of naive

B-cells to IgA secretion; the mechanism of IgA induction requires the cooperation of IL10 and TGF-beta. Soluble CD40 inhibits T-cell-dependent B-cell proliferation.

Monoclonal antibodies against CD40 mediate a variety of effects on B-lymphocytes, including induction of intercellular adhesion (via CD11a/CD18 (LFA-1)), short- and long-term proliferation, differentiation and enhanced tyrosine phosphorylation of proteins. Germinal center centrocytes are prevented from undergoing cell death by apoptosis by activation through CD40 and antigen receptors.

In human resting B-cells expression of CD40 is induced by IL4. Treatment of human B-cells with IL6 leads to the phosphorylation of the intracellular CD40 domain. CD40 does not, however, function as a receptor for IL6. In activated human B-cells the synthesis of IL6 is induced by treatment of the cells with monoclonal antibodies directed against CD40, suggesting that CD40 participates in signal transduction mechanisms dependent on IL6.

Some limited sequence homologies have been found with receptors for Nerve Growth Factor, TNF-alpha and CD27 and it has been assumed that CD40 may be involved also in modulating the biological activity of these and other cytokines.

CD40 has biological functions also in non-immune cells although these are still largely unknown. CD40 ligation has been shown to induce cell death by apoptosis in transformed cells of mesenchymal and epithelial origin. In part these processes are mediated through the death domain present in the cytoplasmic domain of CD40.

A particularly useful ligand for CD40 is CD154. CD154 ("CD40 ligand"; human protein 29.3 kDa, 261 amino acids) is a member of the TNF family of proteins. The human protein shows 82.8% and 77.4% identity at the cDNA and protein level, respectively, with a similar protein isolated from murine EL4 thymoma cells. Both proteins are the ligands for the CD40 cell surface antigen expressed on resting B-cells. The human gene encoding CD154 maps to chromosome Xq26.3-q27. Nucleotide sequences encoding the native CD40 ligands of various species are provided by, e.g., Genbank Accession Nos. X67878, X96710, X68550, X65453, Z48469, and L07414. Amino acid sequences of the CD154 molecules of various species are provided, e.g., by Entrez protein database Accession Nos. 1705713, 231718, 560693, 3047129, 116000, 1518170, 38412, 109639, 1083014, 38484, and 37270.

CD154 is naturally synthesized as a transmembrane polypeptide. Nevertheless, a biologically active soluble fragment of human CD154 has been described (Pietravalle et al, 1996, J Biol Chem 271:5965-5967). Mazzei et al., (1995, J Biol Chem 270:7025-7028) identified a biologically active soluble fragment of CD154 as a homotrimer of polypeptides consisting of amino

acids Glu 108 through Leu 261 of intact transmembrane CD154. Graf et al., (1995, Eur J Immunol. 25:1749) describe another active fragment consisting of the C-terminal fragment produced by proteolyttic cleavage at Met 113. Aruffo et al., disclose soluble forms of CD154 and their use to stimulate B cells *in vitro* in U.S. Pat. 5, 540, 926. In the present invention, particularly useful ligands for CD40 include polypeptides that comprise a sequence as set forth in SEQ ID NO. 2 of the '926 patent, from amino acid residues 47 to 261. These residues are comprised by the extracellular domain of human CD154.

Another particularly useful type of ligand for CD40 is an antibody to CD40. Examples of such antibodies include the monoclonal antibodies designated product numbers MCA1143 and MCA1590 of Harlan Bioproducts for Science (Indianapolis, IN); monoclonal antibodies designated catalog numbers P61640F (produced by clone 14G7), P42374M (produced by clone MAB89), P61046M (produced by clone BL-C4), and P54486M (produced by clone B-B20) of Biodesign International (Kennebunk, ME); monoclonal antibody designated catalog number 05-422 (produced by clone 626.1) of Upstate Biotechnology (Lake Placid, NY); monoclonal antibody designated catalog number 3601 (produced by clone S2C6) of Mabtech (Nacka, Sweden); monoclonal antibodies designated catalog numbers RDI-CBL486 (produced by clone BB20), RDI-M1691clb (produced by clone CLB-14G7), RDI-mCD40-323 (produced by clone 3/23) of Research Diagnostics (Flanders, NJ); monoclonal antibodies described in Schwabe et al, 1997, Hybridoma 16:217-226; monoclonal antibodies described in Bjorck et al, 1994, Immunology 83:430-437; monoclonal antibody G28-5 described by Ledbetter et al, 1994, Circ Shock 44:67-72; and monoclonal antibodies described in Buske et al, 1997, Exp Hematol 25:329-337.

Antigens Useful According to the Invention

The term "antigen" as used herein refers to a molecule which can initiate a humoral and/or cellular immune response in a recipient of the antigen. The antigen is preferably an agent that causes a disease for which a vaccination would be advantageous treatment. The antigen portion of the fusion polypeptide is preferably at least 8 amino acids, and is preferably no longer than 25 amino acids, and preferably does not include more than 10 contiguous amino acids of an opsonin, or a lectin binding domain of a eukaryotic intercellular adhesion molecule, or a reporter molecule such as b-galactosidase. As used herein, a "lectin binding domain" refers to a carboxy-terminal carbohydrate recognition domain of a protein, for example exon 4, nucleotides 439-813 of human mannose binding protein. "Complement-fixing domain" refers to a collagen-like segment having a repeated pattern of Gly-X-Y (where X and Y represent any amino acid) similar to those of

non-fibrillar collagen genes. The structure is consistent with those of an effector region which interacts with complement components; for example, exon 2, nucleotides 253-369 of human mannose binding protein.

Antigens can be any type of biologic molecule including, for example, simple intermediary metabolites, sugars, lipids, and hormones as well as macromolecules such as complex carbohydrates, phospholipids, nucleic acids and proteins. According to the invention, cells that comprise or are attached to a molecule that can elicit an immune response are also considered antigens. Common categories of antigens include, but are not limited to, viral antigens, bacterial antigens, fungal antigens, protozoal and other parasitic antigens, tumor antigens, antigens involved in autoimmune disease, allergy and graft rejection, and other miscellaneous antigens.

Examples of viral antigens include, but are not limited to, retroviral antigens such as retroviral antigens from the human immunodeficiency virus (HIV) antigens such as gene products of the *gag*, *pol*, and *env* genes, the Nef protein, reverse transcriptase, and other HIV components; hepatitis viral antigens such as the S, M, and L proteins of hepatitis B virus, the preS antigen of hepatitis B virus, and other hepatitis, e.g., hepatitis A, B, and C, viral components such as hepatitis C viral RNA; influenza viral antigens such as hemagglutinin and neuraminidase and other influenza viral components; measles viral antigens such as the measles virus fusion protein and other measles virus components; rubella viral antigens such as proteins E1 and E2 and other rubella virus components; rotaviral antigens such as VP7sc and other rotaviral components; cytomegaloviral antigens such as envelope glycoprotein B and other cytomegaloviral antigen components; respiratory syncytial viral antigens such as the RSV fusion protein, the M2 protein and other respiratory syncytial viral antigen components; herpes simplex viral antigens such as immediate early proteins, glycoprotein D, and other herpes simplex viral antigen components; varicella zoster viral antigens such as gpI, gpII, and other varicella zoster viral antigen components; Japanese encephalitis viral antigens such as proteins E, ME, MENS1, NS1, NS1NS2A, 80%E, and other Japanese encephalitis viral antigen components; rabies viral antigens such as rabies glycoprotein, rabies nucleoprotein and other rabies viral antigen components. *See* Fundamental Virology, Second Edition, eds. Fields, B.N. and Knipe, D.M. (Raven Press, New York, 1991) for additional examples of viral antigens.

Bacterial antigens which can be used in the compositions and methods of the invention include, but are not limited to, pertussis bacterial antigens such as pertussis toxin, filamentous hemagglutinin, pertactin, FIM2, FIM3, adenylate cyclase and other pertussis bacterial antigen components; diptheria bacterial antigens such as diptheria toxin or toxoid and other diptheria

bacterial antigen components; tetanus bacterial antigens such as tetanus toxin or toxoid and other tetanus bacterial antigen components; streptococcal bacterial antigens such as M proteins and other streptococcal bacterial antigen components; gramnegative bacilli bacterial antigens such as lipopolysaccharides and other gramnegative bacterial antigen components; Mycobacterium tuberculosis bacterial antigens such as mycolic acid, heat shock protein 65 (HSP65), the 30kDa major secreted protein, antigen 85A and other mycobacterial antigen components; Helicobacter pylori bacterial antigen components; pneumococcal bacterial antigens such as pneumolysin, pneumococcal capsular polysaccharides and other pneumococcal bacterial antigen components; haemophilus influenza bacterial antigens such as capsular polysaccharides and other haemophilus influenza bacterial antigen components; anthrax bacterial antigens such as anthrax protective antigen and other anthrax bacterial antigen components; rickettsiae bacterial antigens such as rompA and other rickettsiae bacterial antigen component. Also included with the bacterial antigens described herein are any other bacterial, mycobacterial, mycoplasmal, rickettsial, or chlamydial antigens.

Fungal antigens which can be used in the compositions and methods of the invention include, but are not limited to, candida fungal antigen components; histoplasma fungal antigens such as heat shock protein 60 (HSP60) and other histoplasma fungal antigen components; cryptococcal fungal antigens such as capsular polysaccharides and other cryptococcal fungal antigen components; coccidiodes fungal antigens such as spherule antigens and other coccidiodes fungal antigen components; and tinea fungal antigens such as trichophytin and other coccidiodes fungal antigen components.

Examples of protozoal and other parasitic antigens include, but are not limited to, plasmodium falciparum antigens such as merozoite surface antigens, sporozoite surface antigens, circumsporozoite antigens, gametocyte/gamete surface antigens, bloodstage antigen pf 155/RESA and other plasmodial antigen components; toxoplasma antigens such as SAG1, p30 and other toxoplasmal antigen components; schistosomae antigens such as glutathioneS transferase, paramyosin, and other schistosomal antigen components; leishmania major and other leishmaniae antigens such as gp63, lipophosphoglycan and its associated protein and other leishmanial antigen components; and trypanosoma cruzi antigens such as the 7577kDa antigen, the 56kDa antigen and other trypanosomal antigen components.

Tumor antigens which can be used in the compositions and methods of the invention include, but are not limited to, telomerase; multidrug resistance proteins such as Pglycoprotein; MAGE1, alpha fetoprotein, carcinoembryonic antigen, mutant p53, papillomavirus antigens, gangliosides or other carbohydrate containing components of melanoma or other tumor cells. It is contemplated by

the invention that antigens from any type of tumor cell can be used in the compositions and methods described herein.

Antigens involved in autoimmune diseases, allergy, and graft rejection can be used in the compositions and methods of the invention. For example, an antigen involved in any one or more of the following autoimmune diseases or disorders can be used in the present invention: diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, StevensJohnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis. Examples of antigens involved in autoimmune disease include glutamic acid decarboxylase 65 (GAD 65), native DNA, myelin basic protein, myelin proteolipid protein, acetylcholine receptor components, thyroglobulin, and the thyroid stimulating hormone (TSH) receptor. Examples of antigens involved in allergy include pollen antigens such as Japanese cedar pollen antigens, ragweed pollen antigens, rye grass pollen antigens, animal derived antigens such as dust mite antigens and feline antigens, histocompatibility antigens, and penicillin and other therapeutic drugs. Examples of antigens involved in graft rejection include antigenic components of the graft to be transplanted into the graft recipient such as heart, lung, liver, pancreas, kidney, and neural graft components. The antigen may be an altered peptide ligand useful in treating an autoimmune disease.

Examples of miscellaneous antigens which can be can be used in the compositions and methods of the invention include endogenous hormones such as luteinizing hormone, follicular stimulating hormone, testosterone, growth hormone, prolactin, and other hormones, drugs of addiction such as cocaine and heroin, and idiotypic fragments of antigen receptors such as Fabcontaining portions of an antileptin receptor antibody.

In one embodiment, the antigen comprises an antigen of a bacterium that infects animals.

In a preferred embodiment, the antigen comprises an antigen of a virus, fungus, parasite, chlamydia, or rickettsia that infects animals. In another preferred embodiment, the antigen is a target of a pathologic autoimmune response. In yet another embodiment, the antigen comprises greater than seven amino acids. In a further embodiment, the antigen is a short peptide comprising no more than twenty amino acids, or preferably no more than twenty-five. It is preferred that the antigen comprise neither more than ten contiguous amino acids of an opsonin, nor a lectin domain of an adhesion molecule, nor a reporter protein such as a portion of beta galactosidase. If the opsonin moiety is derived from a2m, it is preferred that the antigen is neither carbonic anhydrase nor a heptapeptide comprising a cleavage site for the Tobacco Etch Virus protease. If the opsonin moiety is derived from mannose binding protein, it is preferred that the antigen is neither CD4 nor a toxic portion of a cytotoxin.

Vectors According to the Invention

Yet another aspect of the invention pertains to vectors, preferably expression vectors, containing nucleic acid molecules of the invention (or a portion or fragment thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded nucleic acid loop into which additional nucleic acid segments can be ligated. Another type of vector is a viral vector, wherein additional nucleic acid segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., nonepisomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant nucleic acid techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adenoassociated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells

to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion polypeptides or peptides, encoded by nucleic acids as described herein.

The recombinant expression vectors of the invention can be designed for expression of the nucleic acid molecules of the invention in prokaryotic or eukaryotic cells. For example, the polypeptides encoded by the nucleic acid molecules of the invention can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or nonfusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion

expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:3140), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione Stransferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible nonfusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 6089). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trplac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al., (1992) *Nuc. Acids Res.* 20:21112118). Such alteration of nucleic acid sequences of the invention can be carried out by standard nucleic acid synthesis techniques.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari. et al., (1987) *Embo J.* 6:229234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933943), pJRY88 (Schultz et al., (1987) *Gene* 54:113123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, the polypeptides encoded by the nucleic acid molecules of the invention can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al., (1983) *Mol. Cell Biol.* 3:21562165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:3139).

In yet another embodiment, the polypeptides encoded by the nucleic acid molecules of the invention are expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMT2PC

(Kaufman et al. (1987), *EMBO J.* 6:187195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue specific regulatory elements are used to express the nucleic acid). Tissue specific regulatory elements are known in the art. Nonlimiting examples of suitable tissue specific promoters include the albumin promoter (liver specific; Pinkert et al. (1987) *Genes Dev.* 1:268277), lymphoid specific promoters (Calame and Eaton (1988) *Adv. Immunol.*. 43:235275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729740; Queen and Baltimore (1983) *Cell* 33:741748), neuron specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:54735477), pancreas specific promoters (Edlund et al. (1985) *Science* 230:912916), and mammary gland specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374379) and the fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537546).

In one embodiment, a recombinant expression vector containing a nucleic acid molecule encoding a fusion polypeptide of the invention is produced. The fusion polypeptides of the invention, i.e., fusion polypeptides which include an antigen portion and a functional opsonin moiety, can be produced by recombinant expression of a first nucleotide sequence encoding an antigen and a second nucleotide sequence encoding a functional opsonin moiety as described, for example, in U.S. Patent No. 5,116,964 to Capon et al., the entire contents of which are hereby incorporated by reference. Fusion polypeptides, which include or do not include a linker amino acid sequence or an amino acid sequence directing secretion of the polypeptide which is not native to either the antigen or the opsonin amino acid sequences, produced by recombinant techniques can be secreted and isolated from a mixture of cells and medium containing the protein or peptide. Alternatively, the fusion polypeptide can be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. Polypeptides and peptides can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins

and peptides. Techniques for transfecting host cells are described in further detail herein.

For example, fusion polypeptides including one or more peptide or polypeptide antigens and one or more functional opsonin moieties can be produced by constructing a fusion gene which includes a nucleotide sequence encoding one or more antigens and a nucleotide sequence encoding one or more functional opsonin moieties. The fusion polypeptide product of the fusion can be expressed and then administered to a recipient mammal, subject etc. as described herein. Libraries of such fusion genes can be generated from microbes, tumor cells, allografts, xenografts, or other genecontaining entities by cloning the entire set of genomic or expressed nucleic acids or any subset thereof into an expression vector which contains one or more nucleotide sequences encoding one or more functional opsonin moieties such that a multitude of fusion genes including one or more opsonins are produced. These fusion genes can also be administered as described herein.

Another aspect of the invention pertains to recombinant host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell may be any prokaryotic or eukaryotic cell. For example, a polypeptides encoded by nucleic acid molecules of the invention can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

<u>Preparation of Host Cells Containing Nucleic Acid Molecules of the Invention via *In Vitro* and *Ex Vivo* Methods</u>

Vector nucleic acid can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride coprecipitation, DEAEdextranmediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook et al. (*Molecular*

Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals. Additional examples of methods of introducing nucleic acid molecules encoding opsonins and/or opsonin/antigen complexes including an antigen and an opsonin are described below. The cells containing the introduced nucleic acid molecules encoding, for example, an opsonin, can themselves be administered to a subject (as the antigen) according to the methods of the invention, e.g., in a vaccine composition.

A. Introduction of Naked Nucleic Acid into Cells *in vitro* or *ex vivo*

1. *Transfection mediated by $CaPO_4$*: Naked nucleic acid can be introduced into cells by forming a precipitate containing the nucleic acid and calcium phosphate. For example, a HEPES buffered saline solution can be mixed with a solution containing calcium chloride and nucleic acid to form a precipitate and the precipitate is then incubated with cells. A glycerol or dimethyl sulfoxide shock step can be added to increase the amount of nucleic acid taken up by certain cells. $CaPO_4$mediated transfection can be used to stably (or transiently) transfect cells and is only applicable to *in vitro* modification of cells. Protocols for $CaPO_4$ mediated transfection can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.1 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.3216.40 or other standard laboratory manuals.

2. *Transfection mediated by DEAEdextran*: Naked nucleic acid can be introduced into cells by forming a mixture of the nucleic acid and DEAEdextran and incubating the mixture with the cells. A dimethylsulfoxide or chloroquine shock step can be added to increase the amount of nucleic acid uptake. DEAEdextran transfection is only applicable to *in vitro* modification of cells and can be used to introduce nucleic acid transiently into cells but is not preferred for creating stably transfected cells. Thus, this method can be used for short term production of a gene product but is not a method of choice for long term production of a gene product. Protocols for DEAEdextranmediated transfection can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.2 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.4116.46 or other standard laboratory manuals.

3. *Electroporation*: Naked nucleic acid can also be introduced into cells by incubating the cells and the nucleic acid together in an appropriate buffer and subjecting the cells to a high voltage electric

pulse. The efficiency with which nucleic acid is introduced into cells by electroporation is influenced by the strength of the applied field, the length of the electric pulse, the temperature, the conformation and concentration of the nucleic acid and the ionic composition of the media. Electroporation can be used to stably (or transiently) transfect a wide variety of cell types and is only applicable to *in vitro* modification of cells. Protocols for electroporating cells can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.3 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.5416.55 or other standard laboratory manuals.

4. *Liposomemediated transfection ("lipofection")*: Naked nucleic acid can be introduced into cells by mixing the nucleic acid with a liposome suspension containing cationic lipids. The nucleic acid/liposome complex is then incubated with cells. Liposome mediated transfection can be used to stably (or transiently) transfect cells in culture *in vitro*. Protocols can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.4 and other standard laboratory manuals. Additionally, gene delivery *in vivo* has been accomplished using liposomes. See for example Nicolau et al. (1987) *Meth. Enz.* 149:157176; Wang and Huang (1987) *Proc. Natl. Acad. Sci. USA* 84:78517855; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278; and GouldFogerite et al. (1989) *Gene* 84:429438.

5. *Direct Injection*: Naked nucleic acid can be introduced into cells by directly injecting the nucleic acid into the cells. For an *in vitro* culture of cells, nucleic acid can be introduced by microinjection. Since each cell is micro injected individually, this approach is very labor intensive when modifying large numbers of cells. However, a situation wherein micro injection is a method of choice is in the production of transgenic animals (discussed in greater detail below). In this situation, the nucleic acid is stably introduced into a fertilized oocyte which is then allowed to develop into an animal. The resultant animal contains cells carrying the nucleic acid introduced into the oocyte. Direct injection has also been used to introduce naked nucleic acid into cells *in vivo* (see e.g., Acsadi et al. (1991) *Nature* 332: 815818; Wolff et al. (1990) *Science* 247:14651468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (e.g., from BioRad).

6. *Receptor mediated DNA Uptake*: Naked nucleic acid can also be introduced into cells by

complexing the nucleic acid to a cation, such as polylysine, which is coupled to a ligand for a cellsurface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson et al. (1992) *J. Biol. Chem.* 267:963967; and U.S. Patent No. 5,166,320). Binding of the nucleic acidligand complex to the receptor facilitates uptake of the nucleic acid by Receptor mediated endocytosis. Receptors to which a nucleic acidligand complex have targeted include the transferrin receptor and the asialoglycoprotein receptor. A nucleic acidligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:21222126). Receptor mediated nucleic acid uptake can be used to introduce nucleic acid into cells either *in vitro* or *in vivo* and, additionally, has the added feature that nucleic acid can be selectively targeted to a particular cell type by use of a ligand which binds to a receptor selectively expressed on a target cell of interest.

Generally, when naked nucleic acid is introduced into cells in culture (e.g., by one of the transfection techniques described above) only a small fraction of cells (about 1 out of 10^5) typically integrate the transfected nucleic acid into their genomes (i.e., the nucleic acid is maintained in the cell episomally). Thus, in order to identify cells which have taken up exogenous nucleic acid, it is advantageous to transfect nucleic acid encoding a selectable marker into the cell along with the nucleic acid(s) of interest. Preferred selectable markers include those which confer resistance to drugs such as G418, hygromycin and methotrexate. Selectable markers may be introduced on the same plasmid as the gene(s) of interest or may be introduced on a separate plasmid.

B. ViralMediated Gene Transfer

A preferred approach for introducing nucleic acid encoding a gene product into a cell is by use of a viral vector containing nucleic acid, e.g., a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of cells receive the nucleic acid, which can obviate the need for selection of cells which have received the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid and viral vector systems can be used either *in vitro* or *in vivo*.

1. *Retroviruses*: Defective retroviruses are well characterized for use in gene transfer for

gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleic acid encoding a gene product of interest inserted into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.109.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:13951398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:64606464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:30143018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:61416145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:80398043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:83778381; Chowdhury et al. (1991) *Science* 254:18021805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:76407644; Kay et al. (1992) *Human Gene Therapy* 3:641647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:1089210895; Hwu et al. (1993) *J. Immunol.*. 150:41044115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.

2. *Adenoviruses*: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431434; and Rosenfeld et al. (1992) *Cell* 68:143155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited *supra*),

endothelial cells (Lemarchand et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:64826486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:28122816) and muscle cells (Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:25812584). Additionally, introduced adenoviral nucleic acid (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced nucleic acid becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; HajAhmand and Graham (1986) *J. Virol.* 57:267). Most replication defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

3. *AdenoAssociated Viruses*: Adenoassociated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.*. (1992) 158:97129). It is also one of the few viruses that may integrate its DNA into nondividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349356; Samulski et al. (1989) *J. Virol.* 63:38223828; and McLaughlin et al. (1989) *J. Virol.* 62:19631973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous nucleic acid is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:32513260 can be used to introduce nucleic acid into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:64666470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:20722081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:3239; Tratschin et al. (1984) *J. Virol.* 51:611619; and Flotte et al. (1993) *J. Biol. Chem.* 268:37813790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, nucleic acid introduced into a cell can be detected by a filter hybridization technique (e.g., Southern blotting) and RNA produced by transcription of introduced nucleic acid can be detected, for example, by Northern blotting, RNase protection or reverse transcriptasepolymerase chain reaction (RTPCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional

activity of the gene product, such as an enzymatic assay. If the gene product of interest to be expressed by a cell is not readily assayable, an expression system can first be optimized using a reporter gene linked to the regulatory elements and vector to be used. The reporter gene encodes a gene product which is easily detectable and, thus, can be used to evaluate the efficacy of the system. Standard reporter genes used in the art include genes encoding betaglactosidase, chloramphenicol acetyl transferase, luciferase and human growth hormone.

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (i.e., express) fusion polypeptides of the invention. Accordingly, the invention further provides methods for producing polypeptides of the invention using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding a polypeptide of the invention has been introduced) in a suitable medium until the polypeptide is produced. In another embodiment, the method further comprises isolating the polypeptide from the medium or the host cell.

Transgenic Animals

The host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which nucleic acid molecules encoding molecules, e.g., polypeptides, of the invention have been introduced. Such host cells can then be used to create nonhuman transgenic animals in which exogenous nucleic acid molecules encoding the polypeptides of the invention have been introduced into their genome or homologous recombinant animals in which endogenous nucleic acid molecules have been altered. Such animals are useful for studying the function and/or activity of the molecules of the invention and for identifying and/or evaluating modulators of the activity of the molecules of the invention. As used herein, a "transgenic animal" is a nonhuman animal, preferably a mammal, more preferably a mouse, in which one or more of the cells of the animal includes a transgene. A transgene is exogenous nucleic acid which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal.

A transgenic animal of the invention can be created by introducing nucleic acid molecules encoding the polypeptides of the invention into the male pronuclei of a fertilized oocyte, e.g., by

micro injection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue specific regulatory sequence(s) can be operably linked to the transgene to direct expression of a polypeptide of the invention to particular cells. Methods for generating transgenic animals via embryo manipulation and micro injection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Patent No. 4,873,191 by Wagner et al. and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the nucleic acid molecule of the invention, e.g., the transgene in its genome and/or expression of the transgene mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding polypeptides of the invention can further be bred to other transgenic animals carrying other transgenes.

Vaccine Compositions

Yet another aspect of the invention features vaccine compositions which include the nucleic acid molecules, the vectors containing the nucleic acid molecules, or the fusion polypeptides of the invention and a pharmaceutically acceptable carrier. These vaccine compositions can provide protection against (used as a prophylactic) infection by the antigen encoded by the nucleic acid molecule or included in the fusion polypeptide of the invention. In addition, the vaccine compositions of the invention can be used to treat (used as a therapeutic) infection by the antigen encoded by the nucleic acid molecule or included in the fusion polypeptide of the invention.

The preparation of vaccine compositions which contain the nucleic acid molecules or the fusion polypeptides of the invention as the active ingredient, is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to infection can also be prepared. The preparation can also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with carriers which are pharmaceutically acceptable and compatible with the active ingredient. The term "pharmaceutically acceptable carrier" refers to a carrier that does not cause an allergic reaction or other untoward effect in subjects to whom it is

administered. Suitable pharmaceutically acceptable carriers include, for example, one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, NacetylmuramylLthreonylDisoglutamine (thrMDP), NacetylnormuramylLalanylDisoglutamine (CGP 11637, referred to as norMDP), NacetylmuramylLalanylDisoglutaminylLalanine2(1'2'dipalmitoylsnglycero3hydroxyphosphoryl oxy)ethylamine (CGP) 19835A, referred to as MTPPE), and RIBI, which contains three components extracted from bacteria, monophosporyl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. Other examples of adjuvants include DDA (dimethyldioctadecylammonium bromide), Freund's complete and incomplete adjuvants and QuilA. In addition, immune modulating substances such as lymphokines (e.g., IFN-gamma, IL2 and IL12) or synthetic IFN-gamma inducers such as poly I:C can be used in combination with adjuvants described herein.

Dosage and Administration

Vaccine or treatment compositions of the invention may be administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories, and in some cases, oral formulations or formulations suitable for distribution as aerosols. In the case of the oral formulations, the manipulation of T Cell subsets employing adjuvants, antigen packaging, or the addition of individual cytokines to various formulation can result in improved oral vaccines with optimized immune responses. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%95% of active ingredient, preferably 2570%.

The nucleic acid molecules or fusion polypeptides of the invention can be formulated into the vaccine or treatment compositions as neutral or salt forms. Pharmaceutically acceptable salts

include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or with organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroides, and such organic bases as isopropylamine, trimethylamine, 2ethylamino ethanol, histidine, procaine, and the like.

Vaccine or treatment compositions are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered depends on the subject to be treated, including, e.g., capacity of the subject's immune system to synthesize antibodies, and the degree of protection or treatment desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 mg to 1000 mg, such as in the range from about 1 mg to 300 mg, and preferably in the range from about 10 mg to 50 mg. Suitable regiments for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and may be peculiar to each subject. It will be apparent to those of skill in the art that the therapeutically effective amount of nucleic acid molecule or fusion polypeptides of this invention will depend, *inter alia*, upon the administration schedule, the unit dose of antigen administered, whether the nucleic acid molecule or fusion polypeptide is administered in combination with other therapeutic agents, the immune status and health of the recipient, and the therapeutic activity of the particular nucleic acid molecule or fusion polypeptide.

The compositions can be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination can include 110 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 14 months for a second dose, and if needed, a subsequent dose(s) after several months. Periodic boosters at intervals of 15 years, usually 3 years, are desirable to maintain the desired levels of protective immunity. The course of the immunization can be followed by *in vitro* proliferation assays of peripheral blood lymphocytes (PBLs) cocultured with ESAT6 or STCF, and by measuring the levels of IFN-gamma released from the primed lymphocytes. The assays can be performed using conventional labels, such as radionucleotides, enzymes, fluorescent labels and the like. These techniques are known to one skilled in the art and

can be found in US Patent Nos. 3,791,932, 4,174,384 and 3,949,064, which are hereby incorporated by reference.

Intramuscular Injection of Naked DNA

Naked DNA refers to DNA that is free from association with proteins or lipids that enhance introduction of the DNA into a host cell. For direct gene transfer of tibialis anterior (TA) muscle in mice, it is optimal to use 6-8 week old mice (weight 19-21 gm). Females give better immune responses for the hepatitis B surface antigen, and this might be true for some other antigens. The choice of mouse strain will also depend on the antigen. Mice should be anaesthetized since awake mice will contract their muscles and squeeze the DNA solution out. We use either sodium pentobarbital anesthesia (75 mg/kg IP) or halothane inhaled anesthetic (e.g., Metofane form Pittman-Moore). After the mice are asleep the hindlimbs are shaved to better reveal the tibial bone and the access to the TA muscle. Shaving off the limbs allows much greater precision and thus reproducibility for the actual injection step.

In preparation for the intramuscular injection, DNA is dissolved in endotoxin-free injectable PBS (not Tris EDTA) and is best at 0.1-2 mg/ml (depending on how immunogenic your protein is and how rapid a response you want).

To inject plasmid DNA use a 27GX3/4" (0.4x20mm) needle attached to a 1 ml tuberculin syringe. A piece of polyethylene tubing (PE 20, ID-0.38 mm) should be fit over the needle such that only 2-3 mm of needle protrudes (basically just the beveled portion should protrude). Fill the syringe with the DNA solution, attach the needle and then slowly fill the needle so that no air bubbles are trapped. The problem of dead volume is simplified using an insulin syringe (see below).

Alternatively, use a U-100 insulin syringe (1 cc or 3/10 cc) which comes with a pre-attached 29G1/2 needle. Polyethylene tubing is used in the same way as described above.

Inject through the skin - the tip of the needle should be about 3 mm lateral to the anterior tibial tuberosity (this is about half way between the knee and the ankle), keeping the needle almost perpendicular to the tibial. Once the needle is in place (push in until the end of the PE tubing rests against the skin with a bit of pressure), inject the 50 l slowly (over approximately 10 sec), hold the needle in place for another 5-10 sec, then remove the needle slowly. If you accidentally pull the needle out before injection, try to reinsert it in the same hole, otherwise you will experience leakage.

Yet another aspect of the invention pertains to methods for modulating an immune response in an animal, e.g., a nonrodent animal, e.g., a nonrodent mammal, to an antigen. These methods

include administering to the animal a nucleic acid molecule or a fusion polypeptide of the invention in an amount and over a period of time effective to modulate an immune response to the antigen in the animal. The term "modulate" as used herein refers to inhibition or activation/stimulation of an immune response to an antigen, a combination of an inhibition and an activation of an immune response (e.g., an inhibition of a humoral immune response and an activation of a cell mediated immune response or vice versa, or an inhibition of a systemic immune reponse and an activation of a secretory immune response or vice versa), or a change in the character of an immune response to an antigen. Assays are provided herein for determining immune response modulation.

Preferred direct targets of the compositions and methods of the invention include phagocytic leukocytes, e.g., cells of monocyte lineage. The term "nonrodent animal" as used herein refers to any animal which is not a rodent, e.g., a mouse or rat. The term "mammal" as used herein refers to a mammal, e.g., a nonrodent mammal. Examples of preferred mammals include domestic mammals kept for purposes of food production, labor, or companionship, and primates, e.g., humans.

The phrase "in an amount and over a period of time effective to modulate an immune response to the antigen in the mammal" refers to a dosage and period of time in which modulation of an immune response in the recipient mammal or recipient subject occurs. In one embodiment, such an immune response can be observed when the recipient subject exhibits, for example, increased resistance to a challenge by the antigen against which the subject has been immunized using the nucleic acid molecules or the fusion polypeptides of the invention. The nucleic acid molecules and the fusion polypeptides of the invention are typically administered to the recipient animal or subject in the form of a vaccine composition by the routes and in the formulations described herein. In addition, the nucleic acid molecules and the fusion polypeptides of the invention, alone or in the form of a vaccine composition, can be administered in combination with other substances which influence immune responses including, but not limited to, cytokines, anaphylatoxins, celldeath inducing molecules, and cell surface molecules.

Yet another aspect of the invention pertains to antibodies reactive with the fusion polypeptides of the invention. The term "antibody" as used herein refers to monoclonal and polyclonal antibodies. For example, by using the fusion polypeptides of the invention as immunogens, antithe fusion polypeptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)).

<u>Assays for Determining Immune Response Modulation According to the Invention</u>

Fusion polypeptides and multichain complexes are useful according to the invention to modulate an immune response in a mammalian, preferably a human, to an antigen or antigens. The polypeptides or complexes are administered and are taken up (i.e., ingested or phagocytosed) by antigen presenting cells.

An "immune response" refers to stimulation/activation of a selected response involving the immune system, or suppression, elimination, or attenuation of a selected response. Thus, to modulate an immune response means that the desired response is more efficient, more rapid, greater in magnitude, and/or more easily induced than when a control protein is administered in an identical fashion.

The following *in vitro* and *in vivo* assays are useful for determining whether an immune response is modulated according to the invention. The assays described in detail below measure stimulation or suppression of cellular or humoral immune responses to an antigen. The antigens referred to in the following assays are representative. It will be apparent to one of skill in the art that an immune response to a selected antigen useful according to the invention may be measured using one or more of the following assays by adapting the assay to that antigen.

I. Detection of Increased Phagocytosis

The following assay may be used in order to determine whether fusion polypeptides or complexes stimulate phagocytosis by antigen presenting cells.

Phagocytosis is examined using monocytes that have been adhered at 37^0 for 30 min in RPMI without added FCS. Sheep erythrocytes are incubated with a candidate opsonin, or its precursor, under conditions such that there are no more than 300 of such molecules, on average, are deposited on each erythrocyte. If a precursor is used, coated erythrocytes are then processed to convert all precursors to the actual candidate opsonin molecule (e.g., See Carlo et al., *J. Immunol.* 123:523-8(1979)). Fresh monocytes are isolated from the subject, and 5×10^4 - 1×10^5 of these cells suspended in 0.25 - 0.5 ml of RPMI medium with 1% BSA. This aliquot is placed in a tissue culture well and incubated for 30 min at 37 C. An excess of coated erythrocytes, suspended at 1.2×10^8 cells/ml, is overlain on the monocytes, the plate is centrifuged for 5 min at 50g, and incubated for 30 min at 37C. Non-ingested material is removed in two hypotonic lysis steps using ice-cold lysing buffer before fixing and staining the adherent cells, and examining the cells under light microscopy. Phagocytosis is quantified by determining the percentage of 100 monocytes ingesting one or more target cells, and the total number of ingested E/100 monocyptes (PI) is recorded.

Stimulation of phagocytosis according to the invention is indicated by a phagocytic index of equal to or greater than 40.

II. Amplification of the immune response usually involves proliferation of particular subpopulations of lymphoid cells that are normally in the resting state.

Proliferative assays have the following applications in clinical studies: (1) Assessment of overall immunologic competence of T cells or B cells as manifested in their ability to respond to polyclonal proliferation signals such as mitogens or anti-CD3 antibodies. Defects in the proliferation may be indicative of fundamental cellular immunologic defect. Low proliferation is often found as a nonspecific secondary effect of chronic disease. (2) Assessment of an individual's response to specific antigens, where low responses are indicative of general or specific immunologic defect. (3) Determination of MHC compatibility by the mixed lymphocyte reaction (MLR).

In addition, proliferative assays are useful for estimating lymphokine production, investigating signal transduction, and assessing growth factor requirements (e.g., lymphokines) for T or B cells. The procedure outlined here measures incorporation of [^{3}H]thymidine into DNA, which usually correlates well with cell growth as measured by changes in cell number. However, when the activation stimulus is toxic, as with chemical activators such as ionomycin plus phorbol myristate acetate (PMA), the burst of new DNA synthesis following activation may not be accompanied with a net increase in viable cells, and, in fact, a decline in cell number may be observed. In this instance, [^{3}H]thymidine incorporation in DNA is more indicative of initial cell stimulation than estimation of cell number. In addition, [^{3}H]thymidine incorporation provides information on cell populations, not on individual cells. Alternate methods, such as flow cytometry may be used for studies requiring that type of information.

Assay For Antigen-Induced T Cell Proliferation

This protocol is designed to test the proliferation of T cells in response to a specific antigen--tetanus toxoid. It can be modified to test T cell proliferation in response to any protein or polysaccharide antigen. Materials: (T cell suspension, autologous antigen-presenting cell suspension (non-T cells), Tetanus toxoid solution (Connaught or State Laboratory Institute of Massachusetts)). (1) Count T cells and adjust to 1×10^6 cells/ml with complete RPMI-10 AB. (2) Treat antigen-presenting cells with mitomycin C (or irradiate with 2500 rad) as in step 2 of one-way MLR protocol. Adjust concentration of antigen-presenting cells to 2×10^5 cells/ml. Antigen-presenting cells can consist of autologous non-T cells or autologous monocytes/ macrophages. (3) Add 100 ul

T cell suspension and 50 ul antigen-presenting cell population to wells; mix just before dispensing. (4) Add 50 ul tetanus toxoid solution to give final concentrations of 0, 1, 5, 10, and 20 ug/ml. Prepare three wells for each dilution. (5) Incubate 6 days in a humidified 37C, 5% CO_2 incubator. (6) Pulse with [^{3}H]thymidine and harvest as described in support protocol.

Assay For Lymphokine-Dependent Cell Proliferation

This protocol assays the lymphokine-dependent proliferation of a lymphocyte population, in this case, the IL-4 dependent proliferation of B cells. Materials: (Tonsil B cell suspension, Anti-IgM cross-linked to Sepharose beads (Bio-Rad), 10,000 U/ml human rIL-4 (Genzyme) in complete RPMI-10). (1) Count tonsil B cells and adjust concentration to 1 x 10^6 cells/ml with complete RPMI-10. (2) Dispense 100 ul of tonsil B cells into each well. Prepare three wells for each experimental condition. (3) Dilute 10,000 U/ml rIL-4 solution 1:10, 1:100, and 1:1000. Add 20 ul of the stock or dilution to appropriate wells to yield 1000 U/ml, 100 U/ml, 10 U/ml, and 1U/ml. Include a control well with no rIL-4. (4) Pipet anti-IgM beads into appropriate wells.

Determine the optimal concentration of beads with pilot experiments. It is best to include several concentrations of beads in each experiment to "bracket" the optimal dose. Prepare wells with tonsil B cells and IL-4 dilutions alone, anti-IgM beads alone, culture medium alone, and all the combinations of IL-4 and anti-IgM bead dilutions. (5) Increase the volume of each well to 200 ul with complete RPMI-10 as necessary. (6) Culture 5 days in a humidified 37^0 C, 5% CO_2 incubator. (7) Pulse with [^{3}H]thymidine and harvest as described in support protocol.

[^{3}H]Thymidine Pulse And Harvest Of Cell Cultures

This protocol is used in conjunction with the preceding protocols to complete the [^{3}H] thymidine incorporation assay. (1) Add 20 ul of 50 uCi/ml [^{3}H]thymidine to each culture (1.0 uCi) at a fixed time before terminating the culture (usually 6 or 18 hr). (2) Harvest cell cultures using an automated multiwell harvester that aspirates cells, lyses cells, and transfers DNA onto filter paper, while allowing unincorporated [^{3}H]thymidine to wash out. Fill and aspirate each row of the microtiter plate ten times to ensure complete cell transfer and complete removal of unincorporated thymidine. Wash each filter strip with 100% ethanol to facilitate drying. Transfer to scintillation vials. For semiautomated harvester, transfer filter dots for each well into scintillation counting vials. For manual transfer, dry filters under lamp and transfer to scintillation vial with forceps. Add

scintillation fluid to each vial. (3) Count samples in scintillation counter until standard deviation is less than 2%. Calculate mean cpm for background cultures and for each experimental condition. There should be less than 20% variation in replicate cultures.

III. Induction And Measurement Of *In Vitro* Antibody Responses

The capacity of the human immune system to mount an antibody response following *in vivo* immunization with a protein or polysaccharide antigen is a revealing indication of the overall integrity of both the B and T cell arms of the immune system. As such, *in vivo* immunization followed by measurement of the antibody response is an appropriate test of immune function in the various acquired and congenital immunodeficiencies and in a host of other conditions affecting the immune system. The following procedures are for *in vivo* immunization and for the measurement of the subsequent immune response using an ELISA technique.

Immuno-Enzymetric Assay For Cytokines Using NIP- And HRPO-Labeled Antibodies

This protocol describes an immunonoenzymetric assay for cytokines using a heterogeneous, noncompetitive immunoassay reaction in which the cytokine is immobilized by a coating antibody bound to a microtiter plate. Unbound material is washed free, and detection is carried out using a different anti-cytokine antibody labeled with the hapten nitroiodophenyl (NIP). This is in turn detected by a horseradish peroxidase (HRPO) conjugate of an anti-NIP antibody, which is revealed with the chromogenic substrate ABTS. In this noncompetitive immunoassay, the immunoassay signal (A_{405}) increases as a direct function of the amount of cytokine present in the sample. Antibodies are prepared as described in Current Protocols in Immunology, 1995, 6.20.2 - 6.20.10.

Coat assay plate. (1) Using a multichannel pipettor, transfer 100 ul of an appropriate dilution of coating antibody into all wells of the assay plate that are to be used. (2) Seal plates with microtiter plate sealer or Parafilm and incubate 2 hr. At 37C. Prepare samples and standards in preparation plate. (3) Dilute each sample (or aliquot of conditioned medium) to be assayed with an equal volume of immunoassay diluent. (4) Pipet less than or equal to 1 ml of each diluted sample to be assayed into the upper chamber of a separate Spin-X microfiltration device. Microcentifuge 5 min. At 10,000 rpm and save the filtrates that collect in the lower chambers. (5) Add 65 ul of each diluted sample to the appropriate well of a preparation plate (i.e., a separate 96-well microtiter plate). (6) Thaw an aliquot of cytokine standard at room temperature and make sure that it is well mixed. Pipet 130 ul into the well of the preparation plate representing the highest concentration on the standard curve. Transfer 65 ul from this well into the next, then continue performing serial 1:1

dilutions in immunoassay diluent so that 65 ul of each concentration represented on the standard curve is placed in appropriate well of the preparation plate. (7) Thaw an aliquot of calibrator at room temperature (if used). Dilute with an equal volume of immunoassay diluent, then pipet 65 ul of diluted calibrator into appropriate well or wells of preparation plate.

Incubate with coating antibody. (8) Remove coated assay plate from incubator. Dip in 2-liter beaker filled with 1 x wash buffer, then invert over sink and flick to remove liquid. Repeat two more times, then bang dry on paper towel. (9) Transfer 50 ul of solution from each well of preparation plate to corresponding well of the assay plate using multichannel pipettor. (10) Seal plate with microtiter plate sealer or Parafilm and incubate 2 hr. at room temperature.

Incubate with detecting antibody. (11) Dilute NIP-labeled detecting antibody specific to cytokine of interest to 1 ug/ml in detecting buffer. (12) Wash assay plate as in step 8. (13) Add 75 ul diluted detecting antibody from step 11 to all wells of assay plate, including unused outer walls. (14) Reseal plate with microtiter plate sealer or Parafilm and incubate 1 hr. at room temperature.

Incubate with HRPO-conjugated anti-NIP antibody. (15) Dilute HRPO-conjugated anti-NIP Mab 1:3000 in detecting buffer. (16) Wash assay plate as in step 8. (17) Add 75 ul of diluted HRPO-labeled anti-NIP antibody from step 15 to all wells of assay plate. (18) Reseal plate with microtiter plate sealer or Parafilm and incubate 1 hr. at room temperature.

Incubate with chromogenic substrate. (19) Wash assay plate as in step 8. (20) Add 100 ul ABTS substrate working solutions to all wells of assay plate. Cover plate and incubate at room temperature until color development reaches desired level (generally until A_{405} for wells containing the highest concentration of standard is between 1.5 and 2). This protocol usually produces an assay that can be read after 30 to 60 min.

Read plate and analyze data. (21) Using microtiter plate reader with computer interface, measure absorbance in all wells at 405 nm in single-wavelength mode or at 405 and 650 nm in dual-wavelength mode. (22) Fit standard data to a curve described by a first-degree (linear), second degree (quadratic), or four-parameter (nonlinear) mathematical function using curve-fitting software. (23) Interpolate absorbance data from unknown cytokine samples to fitted standard curve, and calculate cytokine concentrations.

IV. Induction of an *in vivo* antibody response provides an approach to the evaluation of the overall integrity of the immune system. In the protocols presented here, diptheria and tetanus toxoids are used as representative protein antigens and pneumococcal polysaccharides are used as representative polysaccharide antigens because of their safety and availability. It should be noted, however, that

the responses elicited by these antigens are likely to be secondary responses because of past vaccination or natural exposure. To obtain a primary response, an unusual antigen such as keyhole limpet hemocyanin should be used.

When antigens are administered by the intramuscular or subcutaneous route, as they are here, a "systemic" immune response is induced and measurement of circulating antibody is most appropriate. It is, however, sometimes of interest to evaluate "local" or mucosal immune responses. In this case, the antigen is given either intranasally to stimulate respiratory lymphoid tissue or orally to stimulate gastrointestinal lymphoid tissue and bronchial washings or intestinal fluids, rather than blood, is assayed for antibody content; in addition, antigens are used that are more appropriate for stimulation of the local/mucosal response (i.e., influenza virus antigen for respiratory responses and cholera toxin for gastrointestinal responses).

In assaying the *in vivo* antibody response, it is important to determine responses to both protein and polysaccharide antigens because these antigens stimulate different components of the immune system. In this regard, the major antibody response to protein antigen is composed of IgG1 and IgG3 subclass antibodies, whereas the major antibody response to polysaccharide antigen is composed of IgG2 subclass antibody.

A variety of immunoassay techniques have been used to measure antibody responses in materials obtained after *in vivo* immunization. Of these, the ELISA assay is perhaps the most useful because it yields a stable, easily measurable, reproducible, and safe readout.

Induction Of *In Vivo* Antibody Responses To Protein/Polysaccharide Antigens

In this protocol antigens are administered by the intramuscular or subcutaneous route and serum is collected for measurement of responses. (1) Draw preimmunized blood sample, allow blood to clot, and separate serum from clot by centrifugation. Store serum at -20C to -70C in appropriately labeled plastic tubes. (2) Inject 0.5 ml of toxoid mixture into an appropriately prepared intramuscular site (deltoid or thigh), taking care not to inject material intravenously. (3) Inject 0.5 ml polyvalent pneumococcal vaccine into an appropriately prepared subcutaneous site, taking care not to inject material intravenously. (4) Draw post-immunization blood samples at desired intervals, usually at 1, 2, and 3 weeks. Separate serum and store at -20C to -70C. (5) After all serum samples are collected, assay samples for presence of antibodies using ELISA.

The ELISA offers a rapid, sensitive, reproducible, nonradioactive method for measuring *in vivo* antibody responses to a variety of antigens, including protein and polysaccharide antigens in

sera obtained from individuals vaccinated with tetanus and diphtheria boosters and the polyvalent pneumococcal polysaccharide vaccine. Assays specific for tetanus, diphtheria and the pneumococcal polysaccharide types I, II, and III are detailed in Current Protocols in Immunology, 1995, Vols. 6 and 7.

The invention is further illustrated by the following exemplification which in no way should be construed as being further limiting. The contents of all cited references (including literature references, issued patents, published patent applications, and copending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXEMPLIFICATIONS

Example 1

A fusion gene incorporating sequences for the pneumococcal antigen pneumolysin and for the alpha chain of the opsonin murine C3b is generated using the following method.

The sequence encoding the alpha chain of murine C3b is amplified by PCR from mouse liver cDNA using an upstream primer corresponding to nt 23012324 of Genbank K02782, and a downstream primer complementary to nt 50185045. The gene for pneumolysin is amplified from pneumococcal DNA using an upstream primer corresponding to nucleotides 207233 of Genbank M17717 and flanked on the 5' end by 12 bases that correspond to nt 50345045 of Genbank K02782 and a downstream primer that corresponds to the sequence complementary to nucleotides 5991622 of M17717 and flanked at the 5' end by a Sph I site. Both products are isolated by agarose gel electrophoresis, eluted using glass beads, and used, in equimolar amounts, in a PCR reaction with excess amounts of the upstream C3b alpha chain primer and the downstream pneumolysin primer. The fusion product is isolated by agarose gel electrophoresis and digested with Sph I. A doublestranded DNA sequence corresponding to the mouse IL2 secretory sequence (nt 48107 of Genbank X01772) and flanked upstream by a singlestranded Hind III overhang is prepared. The mammalian expression vector pcDNA3 is digested with Hind III and Sph I, and the fragments are ligated into the plasmid so that the CMV promoter is upstream of the IL2 signal sequence, which is upstream of the C3b alpha chain/pneumolysin fusion gene.

Example 2

A fusion gene incorporating sequences for the pneumococcal antigen pneumolysin and for

the opsonin murine C3 is generated using the following method.

The sequence encoding murine C3 is amplified by PCR from mouse liver cDNA using an upstream primer corresponding to nt 5778 of Genbank K02782, and a downstream primer complementary to nt 50185045. The gene for pneumolysin is amplified from pneumococcal DNA using an upstream primer corresponding to nucleotides 207233 of Genbank M17717 and flanked on the 5' end by 12 bases that correspond to nt 50345045 of Genbank K02782 and a downstream primer that corresponds to the sequence complementary to nucleotides 5991622 of M17717 and flanked at the 5' end by a Sph I site. Both products are isolated by agarose gel electrophoresis, eluted using glass beads, and used, in equimolar amounts, in a PCR reaction with excess amounts of the upstream C3 primer and the downstream pneumolysin primer. The fusion product is isolated by agarose gel electrophoresis and digested with Sph I. The mammalian expression vector pcDNA3 is digested with Hind III, blunted with Klenow, and then digested with Sph I, and the fusion gene is ligated into the plasmid so that it is downstream of the CMV promoter.

In Examples 1 and 2, fusion constructs comprising pneumolysin are useful for vaccinating an animal against infection with S. pneumoniae, e.g., pneumonia, meningitis, bacteremia, pericarditis, otitis media, or osteomyelitis. Efficacy of the vaccine, administered prophylactically or therapeutically, can be evidenced by a decreased incidence or prevalence of pneumococcal disease, a lower case-mortality rate, fewer complications, or a shorter mean duration of disease (e.g., reduction from 10 to 5 days) in a population.

Example 3

A fusion gene incorporating sequences for the antigen chicken lysozyme and for the opsonin murine mannose binding protein A (MBP) and for a Gly_3 linker is generated using the following method.

The sequence encoding MBP is amplified by PCR from mouse liver cDNA using an upstream primer corresponding to nt 121142 of Genbank S42292 and a downstream primer complementary to nt 818837 and flanked on its 5' side by 12 residues of polyC. Lysozyme is amplified from chicken embryo cDNA using an upstream primer corresponding to nt 82102 of Genbank V00428 and flanked on its 5' side by 12 residues of polyG, which is itself flanked 5' by a sequence corresponding to nt 829837 of S42292, and a downstream primer complementary to nt 450468 of V00428 flanked on the 5' side by an Xba I site. Both products are isolated by agarose gel electrophoresis, eluted using glass beads, and used, in equimolar amounts, in a PCR reaction with excess amounts of the upstream MBP primer and the downstream lysozyme primer. The fusion

product is isolated by agarose gel electrophoresis and digested with Xba I. The mammalian expression vector pcDNA3 is digested with Hind III, blunted with Klenow, and then digested with Xba I, and the fusion gene is ligated into the plasmid so that it is downstream of the CMV promoter.

Example 4

A fusion gene incorporating sequences for an immunodominant peptide of chicken lysozyme and for the opsonin murine mannose binding protein A (MBP) is generated using the following method.

The sequence encoding MBP is amplified by PCR from mouse liver cDNA using an upstream primer corresponding to nt 121-142 of Genbank S42292 and a downstream primer complementary to nt 818-837. A double-stranded oligonucleotide is obtained corresponding to nt 196-237 of Genbank V00428 and flanked on its downstream side by a single-stranded Xba I overhang. The pcDNA3 plasmid is digested with Hind III, blunted with Klenow, and digested XbaI. The MBP sequence is ligated to the vector and the product is isolated by agarose gel electrophoresis and glass beads elution. The lysozyme peptide encoding sequence is ligated into the latter product, and, using restriction digest analysis and DNA sequencing, clones are identified in which a single MBP gene in sense orientation is immediately downstream of the promoter and upstream of the lysozyme peptide gene.

Example 5

In this example, the APC binding domain is located at the carboxy terminus of the fusion polypeptide and the antigen is located at the amino terminus of the fusion polypeptide.

A fusion gene incorporating sequences for an immunodominant peptide of chicken lysozyme and for the APC binding moiety of the opsonin murine a2macroglobulin is generated using the following method.

The sequence encoding a2m is amplified by PCR from mouse liver cDNA using an upstream primer corresponding to nt 3683-3709 of Genbank M93264 and a downstream primer complementary to nt 4510-4537 and flanked on its 3' side by an Xho I site. A double-stranded oligonucleotide is obtained corresponding to nt 196-237 of Genbank V00428 and flanked on its downstream side by a sense-strand C paired with an antisense-strand G, followed by a sense-strand G paired with an antisense-strand C, followed by a sense-strand Apa I overhang. A double-stranded DNA molecule corresponding to the mouse IL-2 secretory sequence (nucleotides 48-107 of Genbank Acc. X01772) flanked upstream by a single-stranded HindIII overhang is prepared. The a2m

fragment is digested with XhoI and ApaI. The pcDNA3 plasmid is digested with Hind III, and digested with XhoI. The three fragments are ligated into the vector.

In Examples 3-5, Chicken lysozyme (hen egg lysozyme) is a well-characterized reporter antigen that can be used to evaluate the immunomodulatory effects of a fusion polypeptide comprising it. Animals can be vaccinated with the plasmid encoding the fusion polypeptide (or the polypeptide itself) or a plasmid encoding the antigen (or the antigen itself). The immune responses to the antigen can then be compared by assays for, e.g., antibody production, lymphocyte proliferation, cell-mediated cytotoxicity, or cytokine production. Another type of assay involves challenging the animals with syngeneic tumor cells transfected with a gene encoding the antigen and examining for a reduced incidence of tumor formation (e.g., from 80% to 40%) in animals receiving the fusion vaccine versus those receiving the antigen vaccine.

Example 6

In this example, the molecule comprises an antigen and two different opsonins. A fusion construct comprising the a2m APC binding site and an immunodominant peptide of chicken lysozyme is prepared in a manner similar to that of Example 5. The gene encoding murine MBPA is then amplified in a manner that allows it to be ligated inframe upstream of the lysozyme peptide. In this case, the MBP moiety provides a secretory signal for the entire fusion polypeptide, which can be inserted into an appropriate expression vector.

Example 7

In this example, three units of the same opsonin are included in one polypeptide. The sequence encoding the alpha' chain of murine C3b is amplified in three ways: in one reaction, the downstream primer includes a sequence that can be annealed inframe to the upstream end of the sequence obtained in a second reaction. The downstream end of the second sequence is designed to anneal inframe to the upstream end of a sequence obtained in a third reaction. The downstream end of the third C3b alpha' encoding sequence is designed to anneal inframe to a sequence encoding a polypeptide chain of telomerase, a tumor antigen. The construct can be cloned into a secretory expression vector which places the murine IL2 secretory sequence inframe upstream of the first C3b.

Example 8

The following example describes an experiment in which mice were immunized with plasmids encoding fusion polypeptides that comprised an antigen and one of two opsonins.

Murine MBP-A (MBP) was amplified from mouse liver cDNA using primers that incorporated 5' EcoRI and 3' NgoMI restriction sites. The downstream primer omitted the stop codon. A cDNA encoding the reporter antigen hen egg lysozyme (HEL) (gift of Dr. E. Unanue) was cloned into the polylinker of pUC19, the 5' end of HEL being ligated to the NgoM1 site of pUC19. The MBP PCR product was restriction digested with EcoR1 and NgoMI and cloned into the pUC19-HEL vector between the EcoRI and NgoMI, in-frame with the HEL cDNA, so that a nucleic acid sequence encoding a fusion polypeptide encoding MBP and HEL was formed. A partial Avr2 and complete Ecor1 digest of puc19-Mbp-HEL, liberated an EcoR1-Avr2 fragment that was cloned into the pCI mammalian expression plasmid (Promega) and thus operably linked to the CMV promoter.

The alpha' chain of C3b was amplified from mouse lung cDNA using an upstream primer that incorporated a 5' EcoRI site upstream of an Avr2 site, which was upstream of the start codon, and a downstream primer that incorporated a 3'NgoMI restriction site. The downstream primer omitted the stop codon. The C3b-alpha' PCR product was digested with EcoRI and NgoMI and cloned into pUC19-HEL as described for MBP. pUC19-C3b alpha'-HEL was then digested with Nhe1 and Avr2 and the insert cloned into a pCI plasmid that comprised a sequence encoding the murine IL-2 secretory signal immediately downstream of the CMV promoter. The latter vector comprised an Nhe1 site immediately downstream of the IL-2 secretory signal sequence that allowed the C3b alpha'-HEL sequence to be inserted in-frame with the signal sequence. Correct orientation of the insert was ascertained by digest with ApaI and HindIII, the former an internal C3b-alpha' restriction site, the latter a site in the pCI vector.

Plasmids were transformed into competent E. coli and suitable quantities of plasmid were purified using the Qiagen Midi Prep kit. Plasmid was suspended in normal saline at 1 mg/ml. Mice were then immunized with either a pCI plasmid with no insert, a pCI-HEL plasmid in which the HEL cDNA is in-frame with and immediately downstream of the murine IL-2 secretory signal sequence, the pCI-MBP-HEL plasmid, or the pCI-C3b alpha' plasmid, according to the following protocol:

Day 0

1. Each animal was anesthetized prior to procedure. After the mouse was asleep, both hindlimbs were shaved over the tibial areas.
2. An orbital sinus bleed was performed. The volume of blood drawn was between 150 and 300 ul. The blood was allowed to clot at room temperature for 4 hours. The clot was removed

and the serum spun at 4000rpm at 4C for 10 min. The supernatant was recovered and stored at -20C until completion of the study.

3. In preparation for injection, each DNA solution was inverted to ensure even dispersion of the solutions. Each solution was administered to a single group, and the three animals in each group received injections of one solution only. A 27GX3/4" (0.4x20mm) needle was attached to a 1 ml tuberculin syringe. A piece of polyethylene tubing (PE 20, ID=0.38 mm) was fitted over the needle so that only 2-3 mm of needle protruded. The syringe was filled with the appropriate solution and the tibialis anterior muscle of the right hindleg was injected with 50 μl of the DNA solution. 50 ul of the same solution was then injected into the tibialis anterior of the left hindleg.

Day 14

Each animal was injected with the same solution it received on Day 0. The protocol for injection was identical to the Day 0 protocol.

Day 28

All animals were euthanized and exsanguinated. The blood was allowed to clot at room temperature for 4 hours. The clot was removed and the serum spun at 4000rpm at 4C for 10 min. The supernatant was recovered and stored at -20C.

Anti-HEL antibodies were measured by ELISA using the following protocol.

50ul of 50ug/ml HEL (Sigma L6876) in sterile PBS pH 7.4 and 0.08% NaN_3 was added to each well of a 96-well plate. In order to minimize variability in the plates, all plates used in the assay were coated with the HEL antigen at the same time under the same conditions. The plates were covered with 96-well plate adhesion tape and incubated overnight at 4°C.

The adhesive tape was removed and the plates washed 4 times with sterile PBS pH 7.4. 50 ul of 1%BSA (Sigma A3059) in sterile PBS pH 7.4 and 0.08% NaN_3 was added to each well and the plate was incubated for 1 hour at 37°C.

The plate was washed 3 times with 1% BSA (Sigma A3059) in sterile PBS pH 7.4 with 0.05% Tween-20 (Mallinckroft) and 0.08% NaN_3. 50 ul of serum at an appropriate dilution was added to each well (triplicate wells). All samples were diluted in 1%BSA (Sigma A3059) in sterile PBS pH 7.4 and 0.08% NaN_3.

The plate was incubated for 1 hour at 37°C and washed four times with 1% BSA (Sigma

A3059) in sterile PBS pH 7.4 with 0.05% Tween 20 (Mallinckroft) and 0.08% NaN_3.

50 ul/well of alkaline-phosphatase conjugated secondary monoclonal antibody, either anti-mouse IgG, anti-mouse IgG1, or anti-mouse IgG2a, also diluted in 1%BSA (Sigma A3059) in sterile PBS pH 7.4 and 0.08% NaN_3, was added and the plate incubated for 1 hour at 37°C.

The plate was washed five times with 1% BSA (Sigma A3059) in sterile PBS pH 7.4 with 0.05% Tween 20 (Mallinckroft) and 0.08% NaN_3. 50ul of p-Nitrophenyl Phosphate (pNPP) tablets (Sigma N9389) dilute one tablet in 5 ml of 0.1 M glycine, 1mM $MgCl_2$, 1mM $ZnCl_2$, pH10.4 (combine 7.51g Glycine, 203 mg $MgCl_2$, 136 $ZnCl_2$ into 1L dH_2O) was added to each well. The plates were incubated at room temperature and optical absorbance at 405 nm periodically measured in a microplate reader. When the highest-diluted sample reached an optical density (OD) of 0.2 the reactions were stopped with the addition of 3 N NaOH as 1 volume base to 4 volume reaction medium.

The absorbance of each well at 405 nm was measured. The absorbance of a well containing only pNPP (no serum or antibody) was subtracted from the OD of each well and mean values were calculated for each set of triplicate wells.

At dilutions ranging from 1:0 to 1:1000, the mean "Day 0" (i.e., pre-immunization) values for all experimental sets lay between 0.055 and 0.088 OD units.

The "Day 28" (post-immunization) values when total anti-HEL IgG was measured were as follows:

Dilution	1:0	1:10	1:100	1:1,000
PCI	0.070	0.059	0.058	0.059
pCI-HEL	0.866	0.319	0.090	0.064
pCI-MBP-HEL	0.093	0.075	0.060	0.060
pCI-C3b alpha'-HEL	0.077	0.063	0.062	0.064

The "Day 28" (post-immunization) values when anti-HEL IgG1 was measured were as follows:

Dilution	1:0	1:10	1:100	1:1,000
PCI	0.0686	0.0610	0.0611	0.0601
pCI-HEL	0.8288	0.1824	0.0746	0.0616

pCI-MBP-HEL	0.0888	0.0643	0.0626	0.0593
pCI-C3b alpha'-HEL	0.0746	0.0697	0.0661	0.0601

The "Day 28" (post-immunization) values when anti-HEL IgG2a was measured were as follows:

Dilution	1:0	1:10	1:100	1:1,000
pCI	0.0660	0.0569	0.0524	0.0538
pCI-HEL	1.2006	0.3068	0.0850	0.0599
pCI-MBP-HEL	0.0986	0.0598	0.0584	0.0557
pCI-C3b alpha'-HEL	0.0691	0.0554	0.0553	0.0537

Thus, administration of a nucleic acid molecule encoding a fusion polypeptide that comprises HEL and an opsonin, i.e., C3b alpha' chain or mannose binding protein, markedly attenuates antibody responses to HEL when compared with administration of a nucleic acid molecule encoding HEL that is not fused to an opsonin.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising an antigen and an APC binding domain of an opsonin in an amount and for a time effective to modulate said immune response.

2. A method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising an antigen and an opsonin in an amount and for a time effective to modulate said immune response.

3. A method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising an antigen and a first portion of an opsonin which when asociated with a second portion of said opsonin forms an APC binding domain or a multichain polypeptide complex comprising (a) a fusion polypeptide comprising an antigen and a first portion of an opsonin which when asociated with a second portion of an opsonin forms an APC binding domain, covalently associated with (b) said second portion in an amount and for a time effective to modulate said immune response.

4. The method of claim 1, 2, or 3 wherein said opsonin is drawn from the group: fibronectin, alpha2macroglobulin (a2m), c-reactive protein (CRP), complement component C1q, complement component C3, complement fragment C3b, complement fragment C4b, mannose binding protein (MBP), conglutinin, and surfactant proteins A and D.

5. The method of claim 1, 2 or 3 wherein said antigen is drawn from the group: an antigen of a bacterium, an antigen of a virus, an antigen of a fungus, an antigen of a parasite.

6. The method of claim 1, 2, or 3 wherein said antigen is drawn from the group: an antigen involved in autoimmune disease, an antigen involved in allergy, an antigen involved in graft rejection.

7. The method of claim 1, 2 or 3 wherein the antigen is a tumor antigen.

8. A method of modulating in an animal an immune response to an antigen, comprising administering to the animal a nucleic acid molecule encoding a fusion polypeptide comprising a secretory signal sequence, an antigen, a first cell binding domain of a ligand for a cell surface polypeptide, and a second cell binding domain of a ligand for a cell surface polypeptide in an amount and for a time effective to modulate said immune response.

9. The method of claim 8 in which the first and second cell binding domains are identical.

10. The method of claim 8 in which the first and second cell binding domains are nonidentical.

11. The method of claim 8 in which the first and second cell binding domains bind to the same cell surface polypeptide.

12. The method of claim 8 in which the first and second cell binding domains bind to different cell surface polypeptides.

13. The method of claim 8 in which neither the first nor the second cell binding domain comprises the antigen.

14. The method of claim 8 in which at least one of the cell binding domains is endogenous in the animal.

15. The method of claim 8 in which at least one of the cell binding domains is an APC binding domain of an opsonin.

16. The method of claim 8 in which the antigen comprises greater than 7 and no more than 25 amino acids.

17. A method of modulating in an animal an immune response to an antigen,

comprising administering to the animal a fusion polypeptide comprising an antigen and an APC binding domain of an opsonin in an amount and for a time effective to modulate said immune response.

18. A method of modulating in an animal an immune response to an antigen, comprising administering to the animal a fusion polypeptide comprising an antigen and an opsonin in an amount and for a time effective to modulate said immune response.

19. The method of claim 17 or claim 18 in which said opsonin is drawn from the group: fibronectin, alpha2macroglobulin (a2m), c-reactive protein (CRP), complement component C1q, complement component C3, complement fragment C3b, complement fragment C4b, mannose binding protein, conglutinin, and surfactant proteins A and D.

20. The method of claim 17 or claim 18 in which said antigen is drawn from the group: an antigen of a bacterium, an antigen of a virus, an antigen of a fungus, an antigen of a parasite.

21. The method of claim 17 or claim 18 in which said antigen is drawn from the group: an antigen involved in autoimmune disease, an antigen involved in allergy, an antigen involved in graft rejection.

22. The method of claim 17 or claim 18 in which the antigen is a tumor antigen.

23. A method of modulating in an animal an immune response to an antigen, comprising administering to the animal a fusion polypeptide comprising an antigen, a first cell binding domain of a ligand for a cell surface polypeptide which is an APC binding domain of an opsonin, and a second cell binding domain of a ligand for a cell surface polypeptide in an amount and for a time effective to modulate said immune response.

24. A method of modulating in an animal an immune response to an antigen, comprising administering to the animal a fusion polypeptide comprising an antigen, an opsonin, and a cell binding domain of a ligand for a cell surface polypeptide in an amount and

for a time effective to modulate said immune response.

25. The method of claim 24 or claim 25 in which said opsonin is drawn from the group: fibronectin, alpha2macroglobulin (a2m), c-reactive protein (crp), complement component C1q, complement component C3, complement fragment C3b, complement fragment C4b, mannose binding protein, conglutinin, and surfactant proteins A and D.

26. The method of claim 23 or claim 24 in which said antigen is drawn from the group: an antigen of a bacterium, an antigen of a virus, an antigen of a fungus, an antigen of a parasite.

27. The method of claim 23 or claim 24 in which said antigen is drawn from the group: an antigen involved in autoimmune disease, an antigen involved in allergy, an antigen involved in graft rejection.

28. The method of claim 23 or claim 24 in which the antigen is a tumor antigen.

29. An isolated nucleic acid comprising a nucleotide sequence encoding a fusion polypeptide comprising an antigen and an APC binding domain of an opsonin, said opsonin being drawn from the group: c-reactive protein (crp), complement component C1q, complement component C3, complement fragment C3b, complement fragment C4b, a collectin, mannose binding protein, conglutinin, and surfactant proteins A and D.

30. The isolated nucleic acid of claim 29 wherein said APC binding domain of an opsonin comprises an opsonin.

31. The isolated nucleic acid of claim 29, said APC binding domain being an APC binding domain of mannose binding protein that does not contain a lectin domain or a complement-fixing domain.

32. An isolated nucleic acid comprising a nucleotide sequence encoding a fusion polypeptide comprising an antigen and an APC binding domain of alpha-2macroglobulin and the antigen is neither a portion of the adenovirus fiber protein nor carbonic anhydrase nor a heptapeptide

which comprises a cleavage site for the TEV protease.

33. The isolated nucleic acid molecule of claim 29 or claim 32 wherein said antigen is drawn from the group: an antigen of a bacterium, an antigen of a fungus, an antigen of a parasite.

34. The isolated nucleic acid molecule of claim 29 in which said antigen is an antigen of a virus.

35. The isolated nucleic acid molecule of claim 32 in which said antigen is an antigen of a virus that infects animals.

36. The isolated nucleic acid molecule of claim 29 or claim 32 in which said antigen is drawn from the group: an antigen involved in autoimmune disease, an antigen involved in allergy, an antigen involved in graft rejection.

37. The isolated nucleic acid molecule of claim 29 or claim 32 in which said antigen is a tumor antigen.

38. The isolated nucleic acid of claim 29 or claim 32 in which the antigen comprises greater than 7 and no more than 25 amino acids.

39. The isolated nucleic acid of claim 29, further comprising a sequence encoding a first portion of an opsonin which when associated with a second portion of said opsonin forms an APC binding domain.

40. The isolated nucleic acid of claim 29 or claim 32 wherein said fusion polypeptide consists essentially of an antigen and an APC binding domain of an opsonin.

41. The isolated nucleic acid of claim 29 wherein the opsonin is mannose binding protein and the antigen is neither CD4 or a cytotoxin.

42. A polypeptide encoded by the nucleic acid of claim 29 or claim 32.

43. A multichain polypeptide complex comprising (a) a fusion polypeptide comprising an antigen and a first portion of an opsonin which when associated with a second portion of an opsonin forms an APC binding domain, covalently associated with (b) said second portion.

44. The multichain polypeptide complex of claim 42 wherein said opsonin is a collectin.

45. A composition comprising the fusion polypeptide of claim 41 admixed with antigen presenting cells.

46. A composition comprising the multichain polypeptide complex of claim 42 admixed with antigen presenting cells.

47. A vector containing the nucleic acid of claim 29 or claim 32.

48. A host cell transfected with the vector of claim 46.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00894

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 133.1, 134.1, 184.1, 192.1, 193.1; 435/69.7, 70.1, 328, 320.1; 530/387.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSCIENCE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,698,679 A (NEMAZEE) 16 December 1997, see entire document.	1-48
Y	US 5,652,342 A (ZIMMERMAN et al.) 29 July 1997, see entire document.	1-48
Y	US 5,668,255 A (MURPHY) 16 September 1997, see entire document.	1-48
Y	WO 96/40941 A1 (CONNAUGHT LABORATORIES LIMITED) 19 December 1996, see entire document.	1-48
Y	FAWELL et al. TAT-mediated Delivery of Heterologous Proteins Into Cells. Proc. Natl. Acad. Sci. USA. January 1994, Vol. 91, pages 664-668, see entire document.	1-48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 APRIL 1999	14 MAY 1999

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer D. Lawrence Tar EVELYN RABIN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOHMANN et al. Macrophage-inducible Expression of a Model Antigen in Salmonella typhimurium Enhances Immunogenicity. Proc. Natl. Acad. Sci. USA. March 1995, Vol. 92, pages 2904-2908, see entire document.	1-48
Y	BAIER et al. Imunogenic Targeting of Recombinant Peptide Vaccines to Human Antigen-Presenting-Cells by Chimeric Anti-HLA-DR and Anti-Surface Immunoglobulin D Antibody Fab Fragments In Vitro. J. Virol. April 1995, Vol. 69, No. 4, pages 2357-2365, see entire document.	1-48

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00894

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00894

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 5/10, 15/13, 15/62, 15/86; C07K 19/00, 16/28; A61K 39/385, 39/395; C12P 21/08

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 133.1, 134.1, 184.1, 192.1, 193.1; 435/69.7, 70.1, 328, 320.1; 530/387.3